EVOLUTIONARY IMPLICATIONS OF MICROSATELLITE VARIATION IN THE *Peromyscus maniculatus* SPECIES GROUP

A Dissertation

by

SCOTT EDWARD CHIRHART

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2003

Major Subject: Biology

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Approved as to style and content by:

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ABSTRACT

Evolutionary Implications of Microsatellite Variation in the *Peromyscus maniculatus* Species Group. (August 2003)

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Given the distribution and probable evolutionary history of the *Peromyscus maniculatus* species group, an interspecific comparison of microsatellite variation among these species would be logically based (at least initially) on primers isolated from the genome of a geographically central population of *P. maniculatus*. Additionally, as the species in the group are recently diverged, reasonably informative microsatellite data are likely to require analysis of a rapid evolving category of microsatellite loci. The initial phase of this research involved the isolation, characterization and assessment of variation for a panel of DNA microsatellites containing perfect dinucleotide repeats from a geographically central population of *P. maniculatus*. Theoretical predictions and empirical studies indicate that phylogenetic analyses based on microsatellite primers isolated from a focal species may be subject to ascertainment biases that can be expected to degrade the efficacy of this approach with increasing phylogenetic depth between the species from which the microsatellites were isolated and those to which these loci are being compared. Results of an analysis of allelic variation at 12 pure, dinucleotide microsatellite loci (isolated from *P. maniculatus*) are reported for samples of all species in the *P. maniculatus* species group and the sister taxon *P. leucopus*.

Examined for the species in the *P. maniculatus* species group for which there is an *a priori* highly corroborated phylogeny, evidence of ascertainment bias was apparent only for one locus that was unique to *P. maniculatus*. Genealogical analyses of the data over all loci yielded inferred relationships that were entirely concordant with the *a priori* corroborated phylogeny for *P. maniculatus*, *P. keeni*, *P. polionotus*, *P. melanotis* and *P. leucopus*. Genealogical analyses of the previously unresolved relationships of *P. keeni* and *P. sejugis* consistently placed these as an independent sister-group between *P. maniculatus* and *P. polionotus*. The geographically improbable sister-group association of *P. keeni* and *P. sejugis* may be the result of an historical ancestral continuity or may reflect large-scale lineage sorting rather than true phylogenetic propinquity. These data suggest that, given the choice of an appropriate focal species, even relatively small sets of pure dinucleotide microsatellites can provide reliable population genetic and systematic implications for taxa with divergence times dating to the Pleistocene.

DEDICATION

My dedications are to three people who have helped me become a better man.

To Ann Chirhart, my loving mother, who taught me the true meaning of the word perseverance. No matter what the odds, you did whatever it took to make sure that Michael and I had everything we needed. I want to express my love and gratitude to you because you have always supported and encouraged me in pursuing my career and my dreams. I want to give you my heartfelt thanks and deepest appreciation.

To Katie Chirhart, my loving wife, who stood by me in good times and bad and tolerated me being almost absent from the first years of our marriage. When I took you as my wife I took you for so much more. You have been and continue to be my hope, my inspiration, my life, my love, and my best friend.

To Ira Greenbaum, my mentor, who through his generous time and commitment has encouraged me to develop my independent thinking and research skills. You continually stimulated my analytical thinking and greatly assisted me with scientific writing. Not all the lessons were wanted or appreciated at the time; however, they have served to make me wiser and to deepen my appreciation for what it really takes to "be a man." Your dedication to students and passion for teaching has given me the best model to substantially influence the career path that I have chosen.

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I also would like to express appreciation to all of my family and friends, especially Dr. Greg Bowling and Katie Connell. Over the past five years Greg and Katie have been witness to the many ups and downs of my own dissertation process. Through their encouraging words and support they got me through many tough times. No one could ask for better friends in life than they have been to me.

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CHAPTER I

INTRODUCTION

Analysis of DNA microsatellites has rapidly become a popular approach for studies of population genetics and evolutionary relationships. The high mutation rate of many microsatellites and the technical ability to select for highly polymorphic loci provide particularly informative data for analyses of population genetic structure, genetic relatedness, patterns of gene flow, and evidence of founder effects, bottlenecks and genetic drift (Weber and Wong 1993; Goldstein and Pollock 1997; Amos 1999). As many microsatellites are conserved over long periods of evolutionary time (Bowen et al. 1993; Fitzsimmons et al. 1995; Rico et al. 1996; Bermingham et al. 1997; Streelman et al. 1998; Martin et al. 2002), microsatellites isolated from one (focal) species are frequently used to examine patterns of genetic variation in various nonfocal species (Rubinzstein et al. 1995, Powell 1997, Petren et al. 1999).

Microsatellites are short (2-5 bp) tandem repeats of DNA sequences that are highly variable even among individuals from the same population (Litt and Luty 1989; Tautz 1989; Weber and May 1989; Amos 1999). Even in relatively small samples, most microsatellite loci are characterized by relatively large numbers of alleles (frequently 10 or more) and heterozygosity estimates above 0.60 (Bowcock et al. 1994;

This dissertation follows the style and format of Molecular Biology and Evolution.

Deka et al. 1995; Primmer et al. 1996). In addition to being highly variable, microsatellites have been found to be characteristic of eukaryotic genomes (Hamada et al. 1982). Microsatellites are generally presumed to be selectively neutral and are, therefore, a preferred marker for high-resolution genetic mapping (Dib et al. 1996, Dietrich et al. 1996).

On average, microsatellite loci occur every 20-30 kb (Stallings et al. 1991) with the most common type of repeat motif being either CA or GT dinucleotides (Tautz et al. 1986). Microsatellites have been classified according to the nature and complexity of the repeats. A perfect (or pure) repeat is one without interruptions, an imperfect repeat contains one or more interruptions, and a compound repeat is characterized by adjacent tandem repeats of different sequence composition (Weber 1990).

Genotypic variation at microsatellite loci can be ascertained using procedures associated with the polymerase chain reaction (PCR), fluorescent PCR primers, and automated sequencing (Weber and May 1989). Microsatellite maps are available for many economically important species including: humans, mice, fruitflies, cows, sheep, deer, chickens, pigs, barley, tomatoes, soybeans, and rice (Postlethwait et al. 1994, Akkaya et al. 1995, Becker and Heun 1995, Crawford et al. 1995, Goldstein and Clark 1995, Broun and Tanksley 1996, Crooijmans et al. 1996, Dib et al. 1996, Dietrich et al. 1996, Ma et al. 1996, Rohrer et al. 1996, Su and Willems 1996, Taramino and Tingey 1996). Markers used to construct these genetic maps provide literally thousands of characterized DNA loci that can potentially be used to examine genetic variation in other species, thus minimizing the time required to isolate markers from each species under

examination. Microsatellite loci are either used to study members of the same species or closely related species, assuming that the DNA sequence is conserved at the primer sites.

The use of microsatellite markers isolated from a particular (focal) species to examine patterns of genetic variation in other (nonfocal) species has become widespread (Rubinstein et al. 1995, Powell 1997, Petren et al. 1999). Implicit in these applications is a thorough understanding of the mutational events that dictate microsatellite evolution (Slatkin 1995a, b; Goldstein et al. 1995, Goldstein and Clark 1995; Feldman et al. 1996). Various studies (Estoup et al. 1995; Fitzsimmons et al. 1995; Garza and Freimer 1996; Zardoya et al. 1996; Angers and Bernatchez 1997), however, suggest that the mutational processes of microsatellites are complex and raise practical questions about the use and interpretation of microsatellites as unbiased markers for studying the population genetics within a species and the derivation of systematic relationships among species. Microsatellites are thought to mutate predominantly by slip-strand mispairing during DNA synthesis, which tends to add or remove a repeat unit to the microsatellite repeat array (Levinson and Gutman 1987, Schlötterer and Tautz 1992). The majority of observed mutations are of a single step (one repeat unit). Studies of 22 observed germline mutations (Weber and Wong 1993) confirmed no mutations larger than 2 repeats. Twenty of these mutations involved a change of a single step for a ratio of 0.91 single-step to two-step mutations. In a subsequent study, Amos et al. (1996) found only a single mutation of larger than one repeat unit out of 15 observed mutations. The general consensus from these studies is that the majority of mutations are one step, but it

remains possible that mutations of much larger sizes occur but are too infrequent to be observed in germline studies (Goldstein et al. 1995).

Microsatellite primers isolated from a focal species have revealed patterns of variation that potentially complicate the interpretation of results in other species. Asymmetrical patterns of allele sizes, levels of polymorphism, and heterozygosity have been observed in various comparisons of variation of orthologous microsatellite loci in focal and nonfocal species (Ellegren et al. 1995; FitzSimmon et al. 1995; Rubinsztein et al. 1995; Forbes et al. 1995; Hutter et al. 1998). Ellegren et al. (1995) proposed that shorter alleles and lower heterozygosity in nonfocal species reflects an ascertainment bias resulting from the cloning and selection of long and highly polymorphic microsatellite loci in the focal species. As microsatellites with longer repeats typically exhibit higher levels of polymorphism, the cloning processes employed are generally designed to select for longer microsatellite loci (Weber 1990). As the orthologous loci in nonfocal species are not selected for length, the latter have independent probabilities of allele length truncation due to a variety of molecular processes (selection-based upper boundary, mutational degeneracy of long repeat lengths, differential rates of microsatellite expansion among alleles of different lengths) that have been hypothesized to constrain the evolutionary elongation of microsatellite repeats (Amos 1999). Therefore, microsatellite ascertainment bias (Ellegren et al. 1995) predicts a reduction in the accuracy with which primers from a focal species identify variation in species at increasing phylogenetic distances from the focal species. Patterns of allelic asymmetry consistent with ascertainment bias have been documented for focal species based

interspecific comparisons of microsatellite loci derived from *Hirundo rustica* (Ellegren et al. 1995), *Chelonia mydas* (Fitzsimmons et al. 1995), *Ovis canadensis* (Forbes et al. 1995), *Bos taurus* and *Capra hircus* (Pepin et al. 1995), *Arabidopsis* (van Treuren et al. 1997), and the *Drosophila obscura* group (Noor et al. 2001). Various authors have suggested that an assessment of allelic asymmetry in microsatellites requires reciprocal comparisons of markers developed from both species (Ellegren et al. 1995; Forbes et al. 1995; Ellegren et al. 1997). In a reciprocal comparison of loci derived from cow and sheep (Ellegren et al. 1997), observations of longer repeats and higher heterozygosity in the focal species were interpreted as resulting from ascertainment bias. Alternatively, Cooper et al. (1998) suggested that the ascertainment bias inferred by Ellegren et al. (1997) reflected sampling error due to the small number of loci examined and the inclusion of loci that were monomorphic in one or both species. A more comprehensive study of reciprocal comparisons of loci derived from cow and sheep (Crawford et al. 1998) suggests that sheep have consistently longer microsatellites and higher heterozygosity regardless of focal species. Hutter et al. (1998) suggested that microsatellite comparisons of Ellegren et al. (1997) and Crawford et al. (1998) were inappropriate for evaluating ascertainment bias and were potentially confounded by the analyses of loci identified from multiple library screens performed under different conditions, in different laboratories, and by the fact that sheep and cattle represent domesticated as opposed to natural populations.

Other studies of interspecific comparisons involving focal and nonfocal species raise additional questions relevant to the hypothesis of the uniform contribution of

ascertainment bias to microsatellite allelic asymmetry between focal and nonfocal species (Rubinsztein et al. 1995, Amos and Rubinsztein 1996). Microsatellites isolated from humans were found to be categorically longer than those in species of non-human primates, regardless of focal species (Rubinsztein et al. 1995). These authors suggested that this pattern of allelic asymmetry is the result of directional evolution. This hypothesis has two tenets. First, the mutational process is such that microsatellites tend to expand more often than contract (Amos et al. 1996; Primmer et al. 1996). Second, mutation rates vary among species with different effective population sizes (N_e). Rubinsztein et al. (1995) and Amos and Rubinsztein (1996) suggested that species characterized by high N_e should have higher levels of variation and experience higher rates of microsatellite evolution. Longer microsatellites in humans were hypothesized to be the result of a comparatively rapid rate of microsatellite evolution reflecting historically higher N_e and the lack of bottleneck and/or founder effects in humans as opposed to the non-human primates. Regardless of the molecular causes(s) of microsatellite asymmetry, the use of primers from one species to assess variation in nonfocal species should be approached with caution.

Another complication in the analysis of microsatellite variation pertains to the extent of molecular and evolutionary convergence. PCR products (amplified from the same set of microsatellite primers) with identical lengths may consist of different sequences or result from different mutations in the same sequence. Although sequencing can readily identify differences in sequences between alleles of the same length, estimating the extent of sequence identity due to different mutations is generally

impractical, as this requires extensive pedigree analysis (Jin et al. 1996). The potential bias due to homoplasy for alleles with identical lengths and sequences is, however, expected to be of greater concern for interspecific comparisons of distantly related focal and nonfocal species than for individual focal species or interspecific comparisons of closely related focal and nonfocal species.

Overall, the available data suggest that reliable population genetic and evolutionary applications of interspecific comparisons of microsatellites isolated from a focal species are constrained by variation in the types of microsatellites employed, the choice of an appropriate focal species, and the phylogenetic distance (total evolutionary time) between the focal and nonfocal species. To test this hypothesis, my study employed an experimental design that included analyses of a uniform category of microsatellite loci in a recently evolved group of mammals (the deer mice in the *Peromyscus maniculatus* species group) for which there are ample data to both specify the appropriate focal species (*P. maniculatus*) and provide a highly corroborated phylogeny (fig.1).

The genus *Peromyscus* (Muridae; Sigmodontinae) constitutes the most successful group of North American small mammals. Distributed from the Atlantic to Pacific seaboards and from the Canadian Northwest Territories to Panama (Hall 1981), at least one of the more than 50 species of *Peromyscus* occurs in nearly all terrestrial habitats within this range. Of the genus *Peromyscus*, no species is more widely distributed or intensely studied than the deer mouse, *P. maniculatus*. Except for the southeastern-most states, mice assigned to *P. maniculatus* range throughout the United States, Canada, and

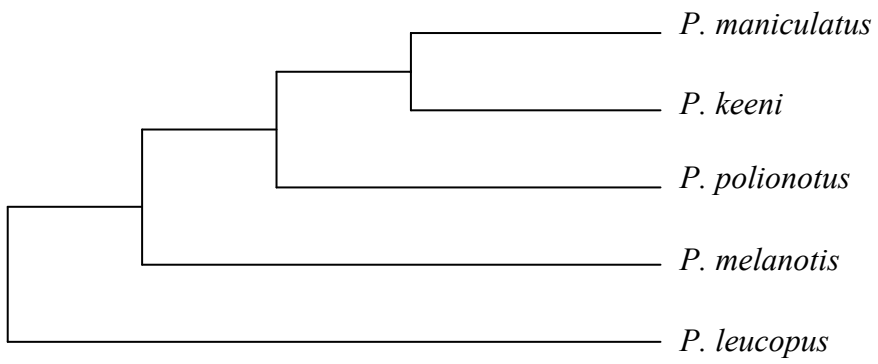

Fig. 1 – The corroborated phylogeny of the species in the *P. maniculatus* species group and its sister taxon, *P. leucopus*.

central Mexico and display both morphological and ecological variation exemplified in the recognition of more than 65 subspecies (Hall 1981). Its widespread distribution, abundance, and overall suitability as a model for laboratory research have made *P. maniculatus* one of the most popular mammals for basic studies of ecology, behavior, physiology, systematics, and evolution. Recently, the significance of deer mouse research has been emphasized by the discovery that these mice are a natural reservoir for a variety of geographic strains of hantavirus (Childs et al. 1994; Morzunov 1998). In addition, deer mice serve as a host for the larval stage of the tick (*Ixodes*), which transmits Lyme disease (*Borrelia*) (Spielman et al. 1985; Caraco et al. 1998).

Following recent revisions (Hogan et al. 1993; 1997; Smith et al. 2000), composition of the *P. maniculatus* group includes the continentally distributed *P. maniculatus* and the geographically peripheral taxa *P. polionotus* (southeastern United States), *P. melanotis* (highlands of central Mexico and southern Arizona), *P. keeni* (Pacific Northwest) and *P. sejugis* (Isla Santa Cruz and Isla San Diego in the Gulf of California). The monophyly of the *P. maniculatus* species group, the systematic relationships of the basal taxa in the group (*P. keeni*, *P. melanotis* and *P. polionotus*), and the sister-group relationship between the *P. maniculatus* and *P. leucopus* species groups (fig. 1) are well supported by phenetic and cladistic analyses of allozymic data (Bowers et al. 1973; Avise et al. 1974a, b; 1979), cladistic analyses of chromosomal banding data (Greenbaum et al. 1978; Yates et al. 1979; Robbins and Baker 1981; Rogers et al 1984; Stangl and Baker, 1984; Smith 1990) and phenetic and cladistic analyses of mtDNA sequence data (Hogan et al. 1997; Engel et al. 1998). Fossil data

(Hibbard 1968) indicate that the *P. maniculatus* group radiated in the late Pleistocene, with divergence between the *P. leucopus* and *P. maniculatus* groups occurring in the mid-Pleistocene (Illinoian Glacial Age, approximately 500,000 years before present). Speciation in the *P. maniculatus* species group presumably occurred by allopatric speciation and was mediated by peripheral isolation from a *P. maniculatus* central stock (Hooper 1968, Greenbaum et al. 1978).

The remaining problem pertaining to the systematics of the *P. maniculatus* group concerns the phylogenetic placement of *P. sejugis* and *P. keeni* (fig. 2). Although both allozymic and chromosomal data associate *P. keeni* and *P. sejugis* with *P. maniculatus* (Avise et al. 1979; Gunn and Greenbaum 1986, Gunn 1988; Hogan et al. 1993, 1997; Smith 2000), resolution of relationships among *P. maniculatus*, *P. sejugis*, and *P. keeni* is equivocal (fig. 2). Further confusion results from preliminary comparisons of sequence data from the ND3/ND4L/ND4 region of the mtDNA (Hogan et al. 1997). Analysis of these sequences for a small number (1 to 2) of individuals suggests a closer relationship of *P. sejugis* and *P. m. coolidgei* from Baja California to *P. keeni*, than to populations of *P. maniculatus* from Colorado and Washington (fig. 3). These data suggest a rather unexpected and geographically complicated pattern; *P. maniculatus* occurs over the entirety of the range between the distribution of *P. keeni* (in

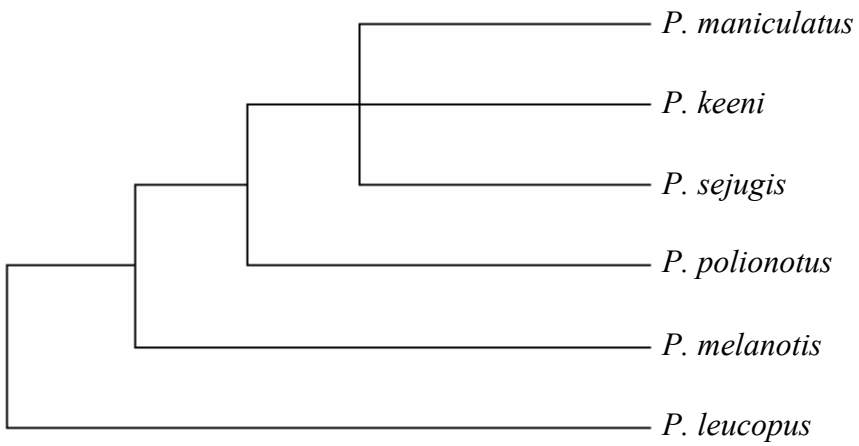

Fig. 2 – Phylogenetic relationships based on chromosome and allozymic data of the species in the *P. maniculatus* species group and its sister taxon, *P. leucopus*.

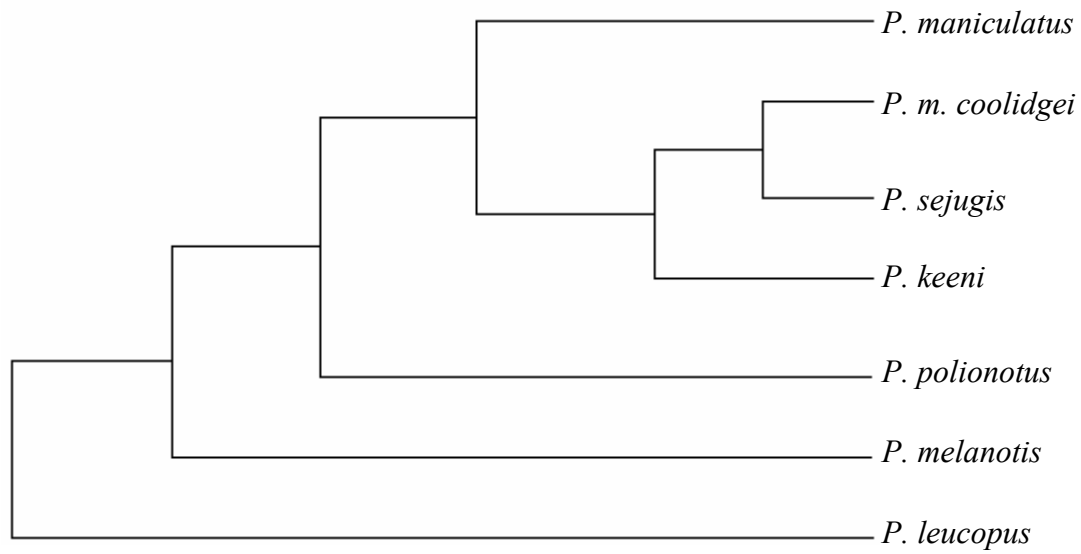

Fig. 3 – Parsimony tree based on mtDNA sequence variation in the *P. maniculatus* species group (Hogan et al. 1997).

the Pacific Northwest) and *P. sejugis* (restricted to two small islands in the Sea of Cortez). Hogan et al. (1997) suggested that the association between *P. keeni* and *P. sejugis* is a result of lineage sorting and therefore reflects coincidental convergence rather than true phylogenetic relatedness.

In order to resolve the relationships among *P. maniculatus*, *P. keeni*, and *P. sejugis*, it is necessary to conduct evolutionary analyses with characters that evolve more rapidly than allozymes or mtDNA. Although DNA microsatellites are similar to allozymes in representing multi-locus, codominant Mendelian characters, the more rapid rate of mutation (Weber and Wong 1993) makes microsatellites more sensitive indicators of population genetic interactions and yields greater genetic divergence over shorter evolutionary times. In the absence of significant ascertainment bias, microsatellite markers are, therefore, likely to provide improved resolution of the evolutionary relationships among recently speciated forms such as *P. maniculatus*, *P. keeni*, and *P. sejugis*.

CHAPTER II

CHARACTERIZATION AND ISOLATION OF MICROSATELLITES FROM *Peromyscus maniculatus*

INTRODUCTION

Given the geographic distribution and probable evolutionary history of the species in the *P. maniculatus* species group, an interspecific comparison of microsatellite variation among these species would be logically based (at least initially) on primers isolated from the genome of a geographically central population of *P. maniculatus*. Ecological, morphological, physiological, chromosomal, and allozymic data all support the conclusion that *P. maniculatus* represents the evolutionary stock from which each of the geographically peripheral species evolved by allopatric speciation via peripheral isolation (Blair 1950, Bowers et al. 1973, Greenbaum et al. 1978, Carleton 1989). Highly polymorphic markers isolated from any of the peripheral isolate species could be expected to exhibit ascertainment bias as a result of independent founder and genetic drift effects associated with the speciation of each peripheral isolate lineage.

Additionally, as the species in the group are recently diverged, reasonably informative microsatellite data are likely to require analysis of a rapidly evolving category of microsatellite loci. As such, the initial phase of this research involved the

isolation, characterization, and assessment of variation for a panel of pure dinucleotide DNA microsatellites from a geographically central population of *P. maniculatus*.

MATERIALS AND METHODS

Phenol/chloroform extractions (Sambrook et al. 1989) were used to isolate genomic DNA from frozen liver tissue of a *Peromyscus maniculatus* (GK 6226, female) from Ellis Co., Kansas. Approximately 30 micrograms (μg) of genomic DNA were partially digested with *Sau*3A1 for one hour at 37°C. The digested DNA was size-separated on a 0.8% agarose gel, and fragments ranging from 300-700 base pairs (bp) were excised from the gel and purified with a Qiagen Gel Extraction Kit (Qiagen, Inc.).

Approximately 50 nanograms (ng) of the size-selected DNA was ligated into 25 ng of dephosphorylated pBluescript SK-plasmid (Stratgene, Inc., LaJolla, CA) that was previously digested with *Bam*H1. Cell transformation was performed using the heat shock method of Sambrook et al. (1989) in a solution of 2 microliters (μl) of ligated plasmid, and 50μl of 10 ng/μl TOP10F' competent cells (Invitrogen, Inc., Carlsbad CA). Recombinant cells were grown on nylon membranes overnight at 37°C, the filters were washed in 10% SDS for 2 minutes, denatured (0.5N NaOH; 1.5M NaCl) for five minutes, neutralized (0.5M Tris; 1.5M NaCl, pH7.4) for 5 minutes, and washed in 2X SSC for five minutes. The filters were then air-dried for one hour and baked at 65°C for 4 hours. Prior to hybridization, the filters were soaked in a buffer (6X SSC; 0.5% SDS;

5X Denhardt's solution) for 1 hour at 65°C. Filters were then probed with $(CA)_{15}$, $(CAG)_{10}$, $(AAT)_{10}$, $(GAAA)_8$, and $(GATA)_8$ oligonucleotides labeled with gamma ^{32}P-[dATP]. Hybridization was performed for 1 hour at 65°C followed by 18 hours at 37°C. The nylon membranes were placed on X-OMAT AR film (Kodak) and exposed overnight at -70°C.

Insert lengths of positive clones were verified by PCR (polymerase chain reaction) amplification with M13 (-21) forward and reverse primers (ABI PRISM™, Perkin Elmer, Inc.). The PCR amplification products with fragment sizes ranging between 300 to 700bp were sequenced on an ABI 377 automated sequencer following the protocol recommended by Applied Biosystems (Applied Biosystems Inc., Foster City, CA). Primer sets were designed from regions flanking the microsatellite repeat. Primer designs were determined using the OLIGO 5.0 computer program (National Biosytems Inc., Plymouth, MN). The forward strand of each forward primer was labeled with one of the three phosphoramidites: 6-Fam (blue), Tet (black), Hex (green) (Applied Biosystems, Inc., Foster City, California) for detection of alleles. As fossil data (Hibbard 1968) indicate that the *P. maniculatus* group is a late Pleistocene radiation, with the divergence of the *P. leucopus* and *P. maniculatus* groups dating to the middle Pleistocene (Illinoian Glacial Age, approximately 500,000 years before present), primer construction was constrained to pure dinucleotide microsatellites as these are characterized by a rate of evolution expected to be informative over the relative short phylogenetic distances among the species being examined (Primmer and Ellegren, 1998).

Including the individual from which the primers were derived, a total of 20 individuals from 2.3 mi. West of Hays, Ellis Co., Kansas (Appendix I), were genotyped using an ABI 310 automated sequencer and primers labeled with one of the three fluorescent dyes (PE Applied Biosystems) (table 1). Cycle sequence reactions contained approximately 100 ng of DNA, 4 µl of 10X PCR Buffer II (PE Applied Biosystems), 2 µl of 25 mm $MgCl_2$, 2.5 µl of 8 mm dNTPs (Amersham Pharmacia Biotech), 0.3 µl of fluorescent labelled (forward) and unlabelled (reverse) primers, and water to a final volume of 25 µl. Reaction conditions for *Pml01, Pml02, Pml04, Pml07, Pml09,* and *Pml10* included an initial 3 min denaturation at 95 °C, followed by 35 cycles at 95 °C for 30 s, annealing temperature (table 1) for 30 s and 72 °C for 25 s. The remaining primers involved a "drop down" or a "touch down" procedure. In this procedure, the annealing temperature phase of the sequence cycle reaction (for any given fluorescent-labeled primer) starts at a higher temperature (for a given number of cycles) and then is reduced for one or more sets of subsequent cycles. A total of 35 cycles was used in all PCR reactions. Forward and reverse primers were run simultaneously in the same tube. The temperatures listed second in table 1 are the second temperatures used in the "touch down" procedure and are only coincidentally aligned next to the reverse primers in the table.

In order to obtain genotypic assignments for each locus, approximately 1.5 µl of each amplified product was mixed with 1.25 µl of deionized formamide, 0.25 µl of loading dye (Applied Biosystems, Inc., Foster City, California), and 0.25 µl of GeneScan-400HD ROX Size Standard (Applied Biosystems, Inc., Foster City,

Table 1
Characterization of the 12 microsatellite loci isolated from *P. maniculatus*. Given are locus identification, fluorescent label (in parentheses), core repeat in the sequenced clone, primer sequence, and PCR amplification conditions.

Locus-Fluores. Label	Cloned repeat	Primer Sequence	Annealing Temp (°C-cycles)
Pml01 (TET)	$(CA)_{18}$	F: CATTCAAGACCTGGCTTTTT R: TGGGTTTCATCAGTGCTTCT	50°C -35 cyc.
Pml02 (HEX)	$(CA)_{20}$	F: GTACCAGGCATGAACATAGT R: GAATAATTTTCCGCTGTGT	52°C -35 cyc.
Pml03 (HEX)	$(CA)_{22}$	F: GCCATTAGTCTATGTGACAG R: GCGATGTACCCAGAAAT	51°C -10 cyc. 49°C -25 cyc.
Pml04 (HEX)	$(CA)_{27}$	F: CATAAGGTGGCTCGGAATCA R: CAGGAAGGGGAAATGACCAT	55°C -35 cyc.
Pml05 (HEX)	$(CA)_{21}$	F: CTGAGCCAAAAGTGGTCCTT R: TGAAGACAGCCCCTCTCTG	57°C-10/55°C-10/53°C-15 cyc.
Pml06 (HEX)	$(CA)_{22}$	F: CAGGGCTGTAGAGGGAGAAC R: ACTGGAGCAGAGGCATTTG	55°C-10/52°C-10/50°C-15 cyc.
Pml07 (TET)	$(CA)_{18}$	F: GCCTCTTGTACCCAGTGAAT R: TCCCATTGGTGTACTTTTTG	52°C -35 cyc.
Pml08 (TET)	$(CA)_{20}$	F: AATGGCTCAGTCCTCTTCC R: GGGTGCTATCAACCTTGTTC	56°C-10/54°C-10/51°C-15 cyc.
Pml09 (TET)	$(CA)_{25}$	F: GAATCCATACACCCATGC R: TTGCTTTTCGTCAAGTTTT	52°C -35 cyc.
Pml10 (FAM)	$(CA)_{21}$	F: CAGCCTGACAAACAGACAG R: TCCCTTAACACACTCACCTC	52°C -35 cyc.
Pml11 (FAM)	$(CA)_{23}$	F: ACCCCCGAGTGCTGAGATT R: TTTGCTGCTTTCCCCAGAGA	57°C-10/55°C-10/53°C-15 cyc.
Pml12 (FAM)	$(CA)_{20}$	F: GCAGCCTGTATTCTCTCACA R: GCCAACCATTTCTTCAAGTG	55°C-10/52°C-10/50°C-15 cyc

California), denatured at 95°C for 5 min, placed on ice for 5 min, and 1.5 µl loaded on an ABI 310 automated sequencer (Applied Biosystems, Inc., Foster City, California). GENESCAN™ and GENOTYPER™ software (Applied Biosystems, Inc., Foster City, California) were used to determine allele sizes by direct comparison against the internal ROX size standard.

RESULTS AND DISCUSSION

A total of 33 positive clones was recovered from approximately 1,500 individual clones yielding a recombinant frequency of 2.20%. Of these 33 positive clones recovered, optimal primer sequences (as indicated by analysis using OLIGO 5.0) could be constructed only for the 12 CA dinucleotide loci (GenBank accession numbers: 329673-329675, 329677-329679, 329680-329686). Each of these 12 primer sets amplified highly polymorphic products (alleles) among the individuals of *P. maniculatus* (table 2). The number of alleles ranged from 10-16 (average = 12.3) per locus; observed heterozygosity (H_O) ranged from 0.75 to 0.95 (average = 0.88) and expected heterozygosity (H_E) ranged from 0.77 to 0.92 (average = 0.87) (table 2). The large range in size of the individual microsatellites (table 2) suggests that in *P. maniculatus* these loci harbor a considerably larger number of alleles than observed in this single populational sample.

The microsatellite variation reported here is much higher than previously reported for *Peromyscus*. Wooten et al. (1999) found 2-5 alleles at five dinucleotide

Table 2
Number of alleles, observed (H_O) and expected (H_E) heterozygosity, and size range for the 12 microsatellites isolated from *P. maniculatus*.

Locus	Number of Alleles	H_O	H_E	Size Range
Pml01	11	0.90	0.85	145-173
Pml02	16	0.85	0.91	197-253
Pml03	12	0.95	0.83	229-263
Pml04	10	0.85	0.85	198-226
Pml05	11	0.85	0.77	186-222
Pml06	14	0.85	0.91	134-172
Pml07	13	0.95	0.92	241-265
Pml08	13	0.75	0.86	209-235
Pml09	11	0.90	0.89	192-246
Pml10	10	0.95	0.81	160-200
Pml11	16	0.85	0.91	220-258
Pml12	11	0.95	0.89	143-175
Average	12.3	0.88	0.87	

microsatellites (average H_O= 0.55) for 10 individuals of *P. polionotus*, and Prince et al. (2002) reported 1-4 alleles for 11 microsatellites (including four complex microsatellite markers) for 8 individuals (average H_O= 0.33) in this same species. The latter markers exhibited an average of 7 alleles per locus in 8 individuals (average H_O= 0.52) of *P. maniculatus* (Prince et al. 2002). Among widespread geographical samples (9-10 individuals/sample) of *P. leucopus*, Schmidt (1999) reported a total range of 11-29 alleles for six microsatellites (including one tetranucleotide marker) with an average of 7.3 alleles per population and an average H_O of 0.58. The lower variation of the microsatellites isolated from *P. polionotus* is consistent with the hypothesis that a history of recent founder effects and genetic drift is likely to characterize microsatellite markers isolated from the peripheral isolate species. The lower variation in the microsatellites isolated from *P. leucopus* may reflect differences in mutation rates among the different categories of microsatellite repeats assessed by Schmidt (1999).

CHAPTER III

THE USE OF MICROSATELLITES IN NONFOCAL SPECIES: ASSESSMENT OF ASCERTAINMENT BIAS AND IMPLICATIONS OF MOLECULAR EVOLUTION IN MICROSATELLITES ISOLATED FROM *Peromyscus maniculatus*

INTRODUCTION

Analysis of DNA microsatellites has rapidly become a popular approach for studies of population genetics and evolutionary relationships. The high mutation rate of many microsatellites and the technical ability to select for highly polymorphic loci provide particularly informative data for analyses of population genetic structure, genetic relatedness, patterns of gene flow, and processes that erode genetic variation (Weber and Wong 1993; Goldstein and Pollock 1997; Amos 1999). As many microsatellites are conserved over long periods of evolutionary time (Bowen et al. 1993; Fitzsimmons et al. 1995; Rico et al. 1996; Bermingham et al. 1997; Streelman et al. 1998; Martin et al. 2002), microsatellites isolated from one (focal) species are frequently used to examine patterns of genetic variation in various nonfocal species (Rubinzstein et al. 1995, Powell 1997, Petren et al. 1999). Several studies, however, have revealed patterns of variation that potentially complicate the interpretation of results from this approach.

Asymmetrical patterns of allele sizes, levels of polymorphism, and heterozygosity have been observed in various comparisons of orthologous microsatellite loci in focal and nonfocal species (Ellegren et al. 1995; FitzSimmon et al. 1995; Rubinsztein et al. 1995; Forbes et al. 1995; Hutter et al. 1998). Ellegren et al. (1995) proposed that shorter alleles and lower heterozygosity in nonfocal species reflects an ascertainment bias resulting from the cloning and selection of long and highly polymorphic microsatellite loci in the focal species. Other proposed explanations for this asymmetry include species-specific differences in mutation rates, differences in the complexity among the loci analyzed, and differences in the demographic and population-genetic histories of the focal and nonfocal species (Blanquer-Maumont and Crouau-Roy 1995; Rubinsztein et al. 1995; Amos and Rubinsztein 1996; Crouau-Roy et al. 1996). Regardless of the cause, systematic asymmetry in variation should be assessed prior to any detailed comparison of orthologous microsatellite loci between focal and nonfocal species. Such an assessment is particularly critical for comparisons of species that are not closely related and/or are suspected to have experienced markedly different histories of populational reductions (genetic bottlenecks) and founding events.

Although reciprocal comparisons of microsatellite loci isolated from all species being compared may provide information relative to determining the extent and potential cause(s) of asymmetry in microsatellite variation, these studies are labor intensive and do not necessarily identify categories or specific loci of value for reliable interspecific comparisons of population genetics or evolutionary relatedness. The latter problem is best approached by assessing the population genetic reliability of a uniform category of

microsatellites isolated from an appropriate focal species in a taxonomic group for which there is a highly corroborated phylogeny (inferred from multiple independent characters) and knowledge of the relative population genetic histories of the inclusive species. In the present study, I analyzed a set of pure dinucleotide microsatellites isolated from the deer mouse (*Peromyscus maniculatus*) to assess the extent of allelic asymmetry among and between species in the *P. maniculatus* species group (and the outgroup species *P. leucopus*) for which the relative population genetic histories are apparent, and the phylogenetic relationships (fig. 1) are highly corroborated.

MATERIALS AND METHODS

Allelic and genotypic variation at 12 DNA microsatellite loci from *P. maniculatus* (Chirhart et al. 2000) was examined from genomic DNA isolated (Sambrook et al. 1989) from frozen liver tissue for 20 individuals each of *P. maniculatus* (Kansas; Ellis Co, 1 mi S, 3 mi W Hays); *P. keeni* (Washington; Okanogan Co; 12.5 mi W Mazama, Lone Fir Campground (n=9), Gray's Harbor Co, 3.0 mi N, 1.0 mi E Grisdale, Satsop Workcamp (n=11)); *P. polionotus* (South Carolina; Lexington Co, 1 mi S Edmund, Bethel Church Field); *P. melanotis* (Mexico: Durango; 44 km SW Ojitos); and *P. leucopus* (Texas: Wichita Co, 8.6 mi N., 1.0 mi. W. Iowa Park, (n=11), Archer Co, 4 mi. N. Windthorst (n=5), Robertson Co, 12 mi. N, 6 mi. W of Girvin TX (n=4)). Detailed information for the specimens examined is presented in Appendix I. Each of the microsatellites examined is a dinucleotide (CA) sequence of between 18 and 27

repeats. Alleles were amplified using the polymerase chain reaction (PCR) with fluorescently labeled primers. PCR protocols and primer pairs were as described in Chirhart et al. (2000) with the re-optimized annealing temperatures and thermo-cycles discussed in Chapter 2. All amplifications were performed on an OMN-E Hybaid thermocycler (Ashford, United Kingdom).

In order to obtain genotypic assignments for each locus, approximately 1.5 μl of each amplified product was mixed with 1.25 μl of deionized formamide, 0.25 μl of loading dye (Applied Biosystems, Inc., Foster City, CA), and 0.25 μl of GeneScan-400HD ROX Size Standard (Applied Biosystems, Inc., Foster City, CA). After denaturing at 95°C for 5 min, the samples were placed on ice for 5 min, and 1.5 μl of each sample was loaded into the gel on an ABI 310 automated sequencer (Applied Biosystems, Inc., Foster City, CA). GENESCAN™ and GENOTYPER™ software (Applied Biosystems, Inc., Foster City, CA) was used to determine allele sizes by direct comparison against the internal ROX size standard.

For the focal (*P. maniculatus*) and nonfocal species, microsatellite variation was determined as the number of alleles, allele size distribution, and expected heterozygosity for each of the 12 microsatellite loci. For each locus, allele lengths and number of repeats were determined by comparison of the PCR product sizes to those of orthologous clones (from *P. maniculatus*) with previously determined sequences (Chirhart et al. 2000). Mean, median, and mode of allele sizes were calculated using SYSTAT (SPSS Inc., Chicago, IL.). Multiple independent chi-square tests were used to test the null hypothesis that mean, median, and modal sizes and numbers of alleles do not differ at

each locus between the focal and nonfocal species. Deviation in the distribution of allele sizes between *P. maniculatus* and each of the nonfocal species was assessed using the Mann-Whitney U (Wilcoxon Rank Sum W) Test. A standard z-test (SYSTAT) was used to determine if the frequency distribution of allele lengths at each locus in each of the nonfocal species had a positive or negative distribution relative to that in *P. maniculatus*. The BIOSYS-1 program (Swofford and Selander 1981) was used to calculate observed and expected heterozygosities. For each locus in each taxon, regression analyses were performed on the microsatellite allele sizes and expected heterozygosities.

To evaluate the possibility of molecular convergence (alleles of similar size differing in sequence complexity within the repeat and/or flanking sequences), a subset of alleles with identical and/or similar sizes was sequenced for each locus in all of the species examined. Each sequence was obtained from the 3' end of the forward to the 5' end of the reverse flanking regions. Where available, sequences for *P. maniculatus* were obtained from the original clones (Chirhart et al. 2000). The remaining sequences were obtained from PCR products of homozygous individuals or from clones generated (SureClone ligation kit, Amersham Pharmacia Biotech) from heterozygous individuals. To remove excess dNTPs and primers from PCR amplification products, these were treated with Exonuclease I and Shrimp Alkaline Phosphatase (EXO-SAP, USB; Cleveland, OH) prior to sequencing. Sequencing reactions were conducted in 5 µl volumes consisting of 5 ng of PCR product for every 100 bp sequenced, 0.8 pM of primer, and 2µl of Big Dye Terminator ready reaction mix (ABI PRISM™, Perkin Elmer, Inc., Foster City, CA). Sequencing parameters were 50 cycles consisting of:

94°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min. Excess unincorporated dyes were removed by separation in sephadex spin columns. Samples were dried, resuspended in 2μl of dextran:formamide solution (1:5), loaded into a 4% polyacrylamide gel and sequenced on an ABI 377 sequencer (Applied Biosystems Inc., Foster City, CA) following the manufacturer's protocol.

To evaluate large-scale ascertainment bias, genealogical trees were produced and compared to the *a priori* established phylogeny for the *P. maniculatus* species group and the outgroup taxon *P. leucopus*. The allelic distributions for each individual and for the individuals grouped according to species were used to compute the following genetic distances: Chord distance (D_C, Cavalli-Sfroza and Edwards 1967), Nei's distance (D_A, Nei et al. 1983) and proportion of shared alleles (D_{PS}, Bowcock et al. 1994). The matrices for each of the genetic distances were subjected to phenetic analyses using the neighbor-joining (Saitou and Nei 1987 in PAUP* Swofford 1999) and Fitch-Margoliash (Fitch and Margoliash 1967 in PHYLIP, Felsenstein 1993) methods.

RESULTS

GenBank BLAST (blastn and blastx) searches of the flanking sequences for all of the loci did not identify association with any previously described introns, coding regions or amino acid sequences. The number of alleles and mean allele sizes for each of the 12 loci in each of the species are presented in table 3. One of the 12 microsatellite primers, *Pml08*, amplified a product only in the individuals of *P. maniculatus*. For this

Table 3
Mean and standard deviation of number of alleles and mean allele lengths (in parentheses) for all taxa examined. Average is the mean number of alleles over all loci examined per species.

Locus	*P. maniculatus*	*P. keeni*	*P. polionotus*	*P. melanotis*	*P. leucopus*	S. D.
Pml01	11 (165.25)	12 (164.30)	12 (162.95)	14 (163.75)	14 (164.05)	1.34 (0.84)
Pml02	16 (237.65)	7 (236.15)	8 (221.00)	13 (235.85)	13 (235.35)	3.78 (6.87)
Pml03	12 (250.65)	13 (264.05)	13 (264.06)	17 (249.55)	13 (248.65)	1.95 (7.90)
Pml04	10 (216.90)	3 (198.55)	10 (216.75)	12 (213.20)	12 (213.85)	3.71 (7.62)
Pml05	11 (213.65)	9 (215.20)	17 (216.40)	10 (207.70)	10 (200.50)	3.21 (6.59)
Pml06	14 (152.75)	15 (152.10)	10 (151.15)	14 (152.80)	12 (156.40)	2.00 (1.99)
Pml07	13 (252.85)	12 (254.80)	14 (251.25)	11 (248.05)	10 (252.25)	1.58 (2.48)
Pml08	13 (220.50)	-	-	-	-	-
Pml09	11 (221.00)	10 (240.75)	9 (236.80)	18 (219.45)	9 (218.55)	5.81 (10.60)
Pml10	10 (180.00)	6 (174.90)	14 (177.90)	12 (177.40)	14 (182.55)	3.78 (2.88)
Pml11	15 (244.25)	8 (236.55)	10 (244.10)	16 (242.10)	13 (249.15)	3.35 (4.55)
Pml12	11 (155.95)	6 (154.40)	5 (146.75)	8 (161.45)	13 (157.10)	3.58 (5.37)
Average	12.3	9.2*	11.1*	13.2*	12.1*	

* Based on 11 loci (excluding *Pml08*).

locus, two additional primer sets (Appendix II) were constructed (using the computer program OLIGO 5.0, National Biosystems Inc., Plymouth, MN) such that the forward and reverse primers were internal or external to the original *Pml08* primer sequences (Chirhart et al. 2000). For subsets (n=5) of the individuals of *P. maniculatus* and each of the nonfocal species, PCR amplifications were conducted using each of these additional primers and all combinations of the forward and reverse primers. For *P. maniculatus*, each of these reactions produced amplification products identical to those obtained from the original primer set. However, these reactions did not yield amplification products for any of the nonfocal species. As a result of the focal species specificity of *Pml08*, quantitative analyses of comparisons between *P. maniculatus* and the nonfocal species necessarily excluded this locus.

The 11 loci present in all species (and *Pml08* in *P. maniculatus*) were polymorphic (Appendix III and Appendix IV) with as few as 3 alleles observed at *Pml04* in *P. keeni* and as many as 18 alleles detected at *Pml09* in *P. melanotis* (table 3). The average number of alleles per species (based on the number of loci amplified) ranged from 9.2 to 13.2 (table 3). The chi-square tests comparing *P. maniculatus* to each of the nonfocal species and to the set of all nonfocal species yielded no significant differences ($p>0.05$) for number of alleles or mean and median allele lengths. Variation in mode of allele lengths (data not shown) was not significant ($p>0.05$) in the comparison of *P. maniculatus* to the set of all nonfocal species. The chi-square tests for mode of allele lengths resulted in significant differences ($0.025<p<0.05$) for the individual comparisons of *P. maniculatus* to both *P. keeni* (loci *Pml03*, *Pml04*, and *Pml09*) and *P. polionotus*

(loci *Pml02* and *Pml09*). However, in two of these five comparisons (*Pml03* in *P. keeni* and *Pml09* in *P. polionotus*), the modal allele length was longer in the nonfocal species than in *P. maniculatus*.

Results of the Mann-Whitney U tests for differences in the distribution of allele sizes revealed significant differences ($0.025<p<0.05$) for three or four loci in each of the nonfocal species as compared to *P. maniculatus* (table 4). However, the comparison of *P. maniculatus* to the set of all nonfocal species yielded no significant differences in the distribution of allele sizes at any of the 11 loci present in all of the species. Chi-square tests for the expectation of a 1:1 ratio of longer versus shorter allele length distributions over all loci in each of the nonfocal species (as compared to *P. maniculatus*) was not significant ($p>0.05$).

The values for observed and expected heterozygosities are presented in table 5. Per-locus observed heterozygosity ranged from 0.55 (for *Pml02* and *Pml11* in *P. polionotus*) to 1.0 (*Pml04* in *P. leucopus*); the corresponding expected heterozygosities ranged from 0.62 (for *Pml04* in *P. keeni*) to 0.94 (for *Pml05* in *P. polionotus*). Chi-square analyses indicated only one significant ($0.025<p<0.05$) difference (*Pml11* in *P. polionotus*) between the observed and expected heterozygosities in each of the taxa examined. Differences in the per-locus and average observed and expected heterozygosities between *P. maniculatus* and the corresponding estimates for each of the nonfocal species and for the combined set of all nonfocal species were not significant (chi-square test, $p>0.05$). Additionally, regression analyses of observed and expected

Table 4
Results of the Mann-Whitney U Test of allele-size distributions between *P. maniculatus* and the nonfocal species examined. + indicates distribution of allele lengths significantly longer than in *P. maniculatus*, – indicates distribution of allele lengths significantly shorter than in *P. maniculatus*, = indicates distribution of allele lengths not significantly different. All levels of significance 0.025<p<0.05.

	P. keeni	*P. polionotus*	*P. melanotis*	*P. leucopus*
Pml01	=	=	=	=
Pml02	=	-	=	=
Pml03	+	+	=	=
Pml04	-	=	-	-
Pml05	=	=	-	-
Pml06	=	=	=	=
Pml07	=	=	-	=
Pml09	-	+	=	=
Pml10	-	=	=	=
Pml11	=	=	=	+
Pml12	=	+	+	=

Table 5
Mean and standard deviation of observed and expected (in parentheses) heterozygosity for all taxa examined.

Locus	*P. maniculatus*	*P. keeni*	*P. polionotus*	*P. melanotis*	*P. leucopus*	S. D.
Pml01	0.90 (0.85)	0.90 (0.89)	0.85 (0.87)	0.85 (0.91)	0.85 (0.87)	0.03 (0.02)
Pml02	0.85 (0.91)	0.70 (0.82)	0.55 (0.67)	0.95 (0.91)	0.85 (0.90)	0.16 (0.11)
Pml03	0.95 (0.83)	0.60 (0.86)	0.60 (0.86)	0.80 (0.93)	0.85 (0.86)	0.16 (0.04)
Pml04	0.85 (0.85)	0.60 (0.62)	0.85 (0.87)	0.95 (0.87)	1.00 (0.91)	0.15 (0.12)
Pml05	0.85 (0.77)	0.75 (0.80)	0.90 (0.94)	0.90 (0.85)	0.80 (0.85)	0.07 (0.06)
Pml06	0.85 (0.91)	0.90 (0.93)	0.60 (0.86)	0.90 (0.93)	0.85 (0.88)	0.13 (0.03)
Pml07	0.95 (0.92)	0.95 (0.90)	0.90 (0.92)	0.95 (0.87)	0.85 (0.84)	0.04 (0.03)
Pml08	0.75 (0.86)	-	-	-	-	-
Pml09	0.90 (0.89)	0.80 (0.87)	0.65 (0.83)	0.85 (0.85)	0.80 (0.82)	0.09 (0.03)
Pml10	0.95 (0.81)	0.65 (0.73)	0.70 (0.91)	0.85 (0.85)	0.80 (0.85)	0.12 (0.07)
Pml11	0.85 (0.91)	0.90 (0.80)	0.55 (0.91)	0.90 (0.91)	0.85 (0.90)	0.17 (0.05)
Pml12	0.95 (0.89)	0.80 (0.75)	0.70 (0.76)	0.80 (0.85)	0.85 (0.91)	0.09 (0.07)

heterozygosities against mean, median, and modal allele lengths (between *P. maniculatus* and each of the nonfocal species) all yielded correlation coefficients that were not significantly different from 0 (regression F Test, $p>0.000$; r^2 for observed heterozygosities ranged from 0.0005 to 0.0258 and for expected heterozygosities ranged from 0.0003 to 0.0202).

Two to four alleles were sequenced for each of the 11 microsatellite loci that amplified in all of the species examined (table 6); the average total flanking sequence (forward + reverse) was 160 bp. The per-species number of alleles sequenced ranged from 21 in *P. melanotis* to 24 in *P. maniculatus*. Sequencing included 14 alleles that were identical in length to alleles in *P. maniculatus* and were present in all or most of the nonfocal species and 13 sets of alleles that represent potential homoplasies as determined by comparison of the allelic distribution to the corroborated phylogeny for the species group. For three loci, individuals of *P. keeni* did not exhibit alleles identical in length to alleles in *P. maniculatus*; at these loci the alleles sequenced included those most similar in length to alleles in *P. maniculatus*. With the exception of differences in the number of CA repeats, the sequences of all alleles were identical.

The neighbor-joining and Fitch-Margoliash analyses of the three estimates of genetic distance (D_C, D_A, and D_{PS}, table 7) yielded entirely concordant results. Conducted using each individual as an independent operational taxonomic unit, all trees correctly clustered all individuals to well-defined groups corresponding to their respective species (fig. 4). All trees produced from the phenetic analyses of the per-species genetic distances (fig. 5) were identical to the *a priori* expected phylogeny.

Table 6
Alleles sequenced at each locus in the focal species (*P. maniculatus*) and each of the nonfocal species. X indicates allele sequenced. Bold indicates sequence obtained from original clones of *P. maniculatus*. – indicates alleles not present in individuals of the respective taxon.

Primer	Allele	*P. maniculatus*	*P. keeni*	*P. polionotus*	*P. melanotis*	*P. leucopus*
Pml01	167	**X**	X	X	X	X
Pml01	169	X	X	X	X	X
Pml02	235	**X**	-	X	X	X
Pml02	237	-	X	-	X	X
Pml02	241	-	X	X	-	X
Pml03	241	**X**	-	-	-	X
Pml03	253	X	X	X	X	X
Pml03	259	X	X	X	X	X
Pml04	194	-	X	-	X	-
Pml04	202	-	X	-	-	-
Pml04	210	X	-	X	-	X
Pml04	224	**X**	-	X	X	X
Pml05	190	X	X	X	X	X
Pml05	220	**X**	X	-	X	-
Pml06	146	X	X	X	X	X
Pml06	148	**X**	X	-	X	X
Pml06	154	X	X	X	X	X
Pml07	243	X	-	X	-	X
Pml07	247	**X**	X	X	X	X
Pml07	257	X	X	X	X	X
Pml09	230	X	-	X	X	X
Pml09	236	**X**	-	X	X	X
Pml09	260	-	X	-	-	-
Pml10	180	**X**	X	X	X	X
Pml10	182	X	X	X	X	-
Pml11	226	-	X	-	-	-
Pml11	244	**X**	-	X	-	X
Pml11	254	X	X	X	X	X
Pml12	147	**X**	X	X	-	-
Pml12	153	X	X	X	X	X

Table 7
Pairwise comparisons of the genetic distances from the allelic data for the 11 microsatellite loci present in all of the species examined. D_{PS} and D_A (in parentheses) above and D_C below the diagonal.

	P. maniculatus	*P. keeni*	*P. polionotus*	*P. melanotis*	*P. leucopus*
P. maniculatus	-	0.483 (0.393)	0.461 (0.458)	0.507 (0.534)	0.472 (0.591)
P. keeni	0.580	-	0.654 (0.420)	0.64 (0.482)	0.652 (0.493)
P. polionotus	0.568	0.657	-	0.594 (0.141)	0.572 (0.173)
P. melanotis	0.598	0.668	0.639	-	0.45 (0.151)
P. leucopus	0.598	0.732	0.661	0.570	-

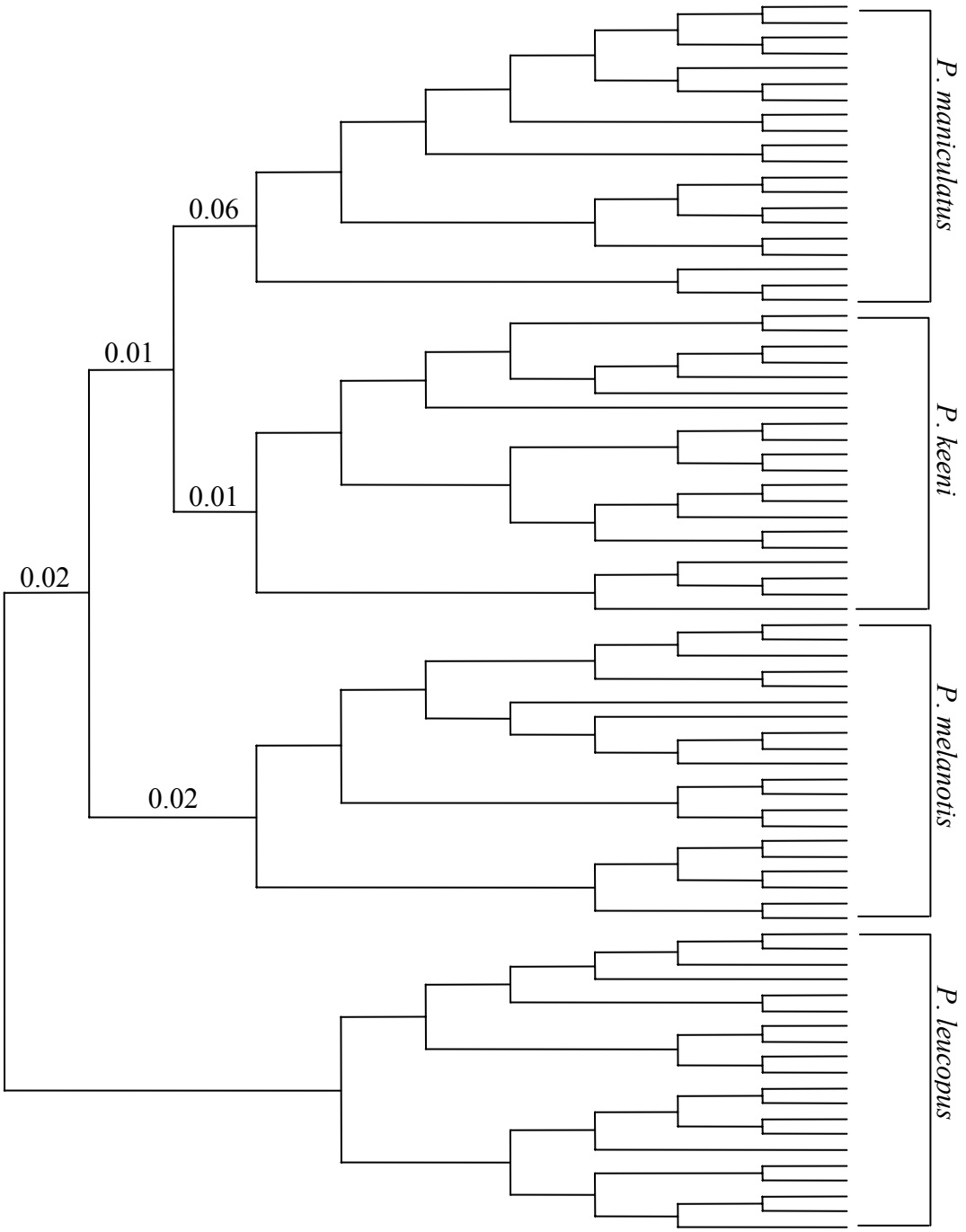

Fig. 4 – Microsatellite-based tree from the neighbor-joining analysis of D_{PS} for the individuals of *P. maniculatus*, *P. polionotus*, *P. melanotis*, and *P. leucopus*. D_{PS} values are shown for the clusters representing each of the species.

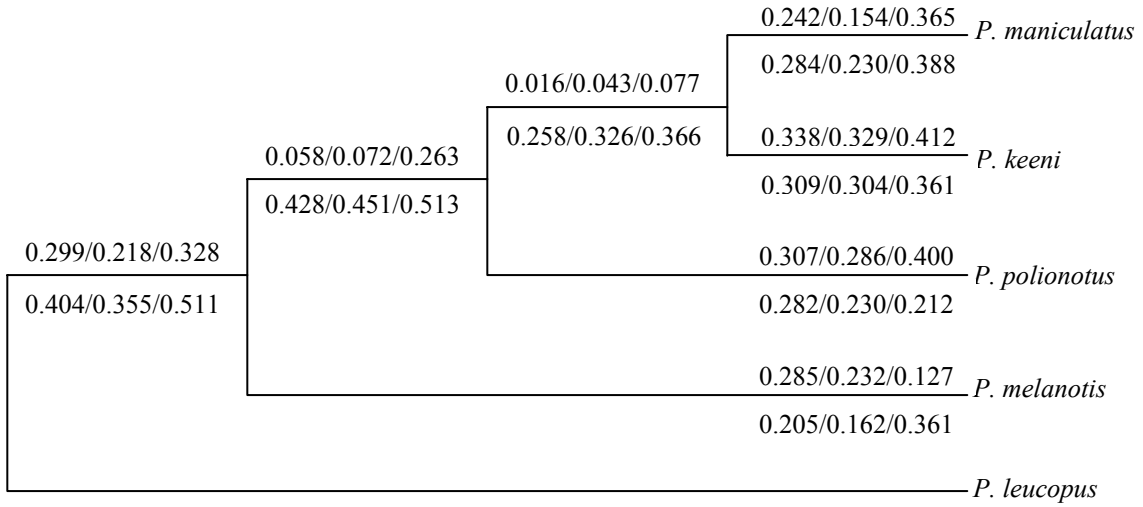

Fig. 5 – Phenogram resulting from the neighbor-joining and Fitch-Margoliash analyses of pairwise genetic distances calculated from the allelic data for *Pml01-07* and *Pml09-12* for *P. maniculatus*, *P. keeni*, *P. polionotus*, *P. melanotis*, and *P. leucopus*. Branch lengths are $D_{PS}/D_A/D_C$ values for the neighbor-joining (above the line) and Fitch-Margoliash (below the line) analyses.

DISCUSSION

The premise of this research is that reliable population genetic and evolutionary applications of interspecific comparisons of microsatellites isolated from a focal species are constrained by variation in the types of microsatellites employed, the choice of an appropriate focal species, and the phylogenetic depth (time since most recent common ancestor) encompassed by the taxonomic group being compared. Although ascertainment bias and differences in the demographic and population genetic histories between focal and nonfocal species have been proposed as alternative explanations for asymmetrical patterns of microsatellite variation, these may actually represent differences reflecting the appropriateness of the specific comparisons addressed such as the choice of uniform category of microsatellite loci, choice of appropriate focal species, and phylogenetic depth for which the microsatellites are applied. Molecular theory (Amos 1999) and the high rate of microsatellite evolution predict that ascertainment bias will result in smaller alleles and lower levels of polymorphism in nonfocal species. The proportion of loci affected by ascertainment bias is expected to increase as a function of increased time since the divergence of the focal and nonfocal species (Ellegren et al. 1995). If the species being compared are closely related, the effects of ascertainment bias for a uniform set (comprising identical repeat motifs) of microsatellites should be inconsequential, and differences in the allelic patterns of variation should accurately reflect population genetic differences and the phylogenetic relatedness of the focal and nonfocal species.

This study was specifically designed to test the latter assumption. Restricting comparisons to a single category of loci, pure dinucleotide repeats, controlled for complications due to differential rates of evolution among categories of microsatellites. The focal and nonfocal species were specifically chosen based on their biogeography, well established phylogenetic relationships, and prior studies reflecting their relative evolutionary and population genetic histories. Following recent revisions (Hogan et al. 1993; 1997; Smith et al. 2000), the *P. maniculatus* species group includes the continentally distributed *P. maniculatus* and the geographically peripheral taxa *P. polionotus* (southeastern United States), *P. melanotis* (highlands of central Mexico and southern Arizona), and *P. keeni* (Pacific Northwest). Ecological, morphological, physiological, chromosomal, and allozymic data all support the conclusion that *P. maniculatus* represents the evolutionary stock from which each of the geographically peripheral species evolved by allopatric speciation via peripheral isolation (Blair 1950, Bowers et al. 1973, Greenbaum et al. 1978, Carleton 1989). The phylogenetic relationships of *P. maniculatus*, *P. keeni*, *P. melanotis* and *P. polionotus*, and the sister-group relationship between the *P. maniculatus* and *P. leucopus* species groups (fig. 1) are all well supported by analyses of biochemical (Avise et al. 1974a,b; 1979; Bowers et al. 1973), cytogenetic (Greenbaum et al., 1978; Yates et al., 1979; Robbins and Baker, 1981; Rogers et al, 1984; Stangl and Baker, 1984; Smith 1990) and molecular (Engel et al. 1998; Hogan et al. 1997) data. Fossil data (Hibbard 1968) indicate that the *P. maniculatus* group is a late Pleistocene radiation and place the divergence of the *P. leucopus* and *P. maniculatus* groups at the mid Pleistocene (Illinoian Glacial Age)

approximately 500,000 years before present. Given these data, *P. maniculatus* represents the most appropriate choice for the focal species upon which to base comparisons of microsatellite variation among the species in the *P. maniculatus* group. As a result of genetic drift and founder effects associated with speciation by peripheral isolation, microsatellite markers isolated from the peripheral species can be expected to exhibit less polymorphism in peripheral focal species than in *P. maniculatus*. Additionally, the well-documented relative chronology of the speciation events within the *P. maniculatus* species group provides a basis for species-specific expectations relative to the extent of the retention of founder and genetic drift effects. This overall experimental design is further validated by the population genetic and biogeographic characteristics of the outgroup taxon *P. leucopus*; this species has an extensive continental distribution and is, therefore, not expected to be subject to reduced genetic variation due to a recent history of genetic drift or bottlenecking.

With the exception of the amplification of *Pml08* only in the focal species, all analyses indicate the lack of confounding effects of ascertainment bias associated with the use of the microsatellites from *P. maniculatus* for the assessment of genetic variation and evolutionary relationships for the species within the *P. maniculatus* group. All available data indicate that *Pml08* is unique to *P. maniculatus*. PCR amplifications, using all combinations of three primer sets (encompassing a total of 226 bases of forward and 84 bases of reverse flanking sequence), amplified identical genotypes of *Pml08* for individuals of *P. maniculatus*, but failed to yield corresponding products from the DNA of any of the nonfocal species. The focal species specificity of *Pml08*

represents a type of microsatellite asymmetry that has not been previously considered. Hypotheses concerning ascertainment bias have uniformly addressed asymmetry in allele size and polymorphism for microsatellite loci shared by both the focal and nonfocal species. In being unique to *P. maniculatus*, *Pml08* represents an absolute bias (relative to nonfocal species) with a molecular basis necessarily different than that proposed to account for ascertainment bias; the selective cloning of long and highly polymorphic microsatellite loci cannot explain the absence of *Pml08* in all of the nonfocal species examined. The focal species specificity of *Pml08* is contrary to the observation that microsatellites are typically conserved over large phylogenetic distances. Focal species-specific microsatellites are apparently uncommon, are not applicable for interspecific comparisons, and pose no difficulty in assessing the reliability of using microsatellites from focal species to assess genetic variation and evolutionary relationships within or among nonfocal species.

Quantitative analyses of the other 11 microsatellites isolated from *P. maniculatus* provided no support for smaller allele sizes and reduced polymorphism in the nonfocal species that would indicate ascertainment bias. Significant differences were not observed in number of alleles or mean and median allele lengths (table 3) or for the comparison of modal allele length between *P. maniculatus* and the set of all nonfocal species. Although marginally significant differences were obtained for modal allele lengths at three loci between *P. maniculatus* and *P. keeni* and at two loci between *P. maniculatus* and *P. polionotus*, two of these differences were for longer modal allele length in the nonfocal species. Similarly, there was no significant difference in allele

size distribution in the comparison of *P. maniculatus* to the set of all nonfocal species and allele size distributions in the individual nonfocal species were mostly not significantly different from *P. maniculatus*. Marginally significant differences at three or four loci in each of the nonfocal species did not conform to the prediction of shorter allele lengths in the nonfocal species. There was no significant deviation from the expectation of a 1:1 ratio of longer versus shorter allele length distributions in the nonfocal species as compared to those in *P. maniculatus*. Of the 56 comparisons of observed and expected heterozygosities in the nonfocal species and in *P. maniculatus*, a significant deviation was obtained at only one locus in a single sample (*Pml11* in *P. polionotus*, table 5). All comparisons of heterozygosity between *P. maniculatus* and the nonfocal species were not significant. Although ascertainment bias is expected to yield positive correlations between heterozygosity and mean, median and modal allele lengths (Hutter et al. 1998), corresponding regression analyses yielded correlation coefficients that were not significantly different from 0. Sequencing of 30 alleles variously shared among and between *P. maniculatus* and the nonfocal species did not implicate molecular convergence as an explanation for the similarity of allele sizes among these species. The sequences of all alleles with identical sizes were identical. Large-scale ascertainment bias can be expected to result in erroneous phylogenetic relationships and/or lack of phylogenetic resolution among sets of nonfocal species and between the focal and nonfocal species. With no significant ascertainment bias, it appears that these microsatellites are phylogenetically informative because the evolutionary relationships interpreted from the data for these microsatellites (fig. 5) are entirely concordant with

the well-established phylogeny for the included species in the *P. maniculatus* group and *P. leucopus*.

The 11 microsatellite loci common to all species appear to have a relatively constant rate of evolution over the timescale represented by the species included in this study. The comparative sequencing analyses indicated that the forward and reverse flanking regions were identical at each locus over all taxa and that the variation in allele lengths at each locus was due only to differences in the number of CA repeats. Mean and median allele lengths were not significantly different ($p>0.05$) for pairwise comparisons among all of the species examined. Significant differences in the distribution of alleles and modal allele lengths among the species probably reflect differences in the population genetic histories (founder effects, bottlenecks and genetic drift) among and between the species since their divergence from a common ancestor. However, the sampling regime was not sufficient to explore comparative population genetics of the species studied. These questions will require studies involving appropriate geographic sampling and substantially larger numbers of individuals.

A radically different conclusion was obtained for the consideration of the molecular evolution of *Pml08*. Empirical data suggest that nascent microsatellites arise by the expansion of short repeat sequences over a long evolutionary timescale until a critical number of repeats has accumulated such that the locus becomes hypervariable (Messier et al. 1996). BLAST searches of the *Pml08* flanking regions failed to identify homology to any previously described intron, coding regions or amino acid sequence. Although not sufficient to identify the specific molecular mechanism, the available data

indicate that *Pml08* may be a novel microsatellite repeat that has evolved and matured (the 13 alleles at *Pml08* are not significantly different ($p>0.01$) from the mean number of alleles at the other 11 microsatellites examined) in *P. maniculatus*. With this explanation of the *Pml08* locus, it would have to be concluded that the evolution of *Pml08* has been extraordinarily rapid because it must have arisen subsequent to divergence of *P. maniculatus* from its sister taxon. As the oldest fossil record of *P. maniculatus* dates to the Wisconsin Age (Hibbard 1968), the origin of *Pml08* can be presumed to have occurred no more than 150,000-200,000 years before present. The data for *Pml08* may document that pure dinucleotide microsatellites can arise rapidly (and possibly accumulate a sufficient number of repeats to become hypervariable) within a species. Another possibility is that homologous regions in the other species in the *P. maniculatus* group and *P. leucopus* have experienced sufficient divergence in the flanking region(s) to obviate amplification of the homolog.

Given the absence of evidence for ascertainment bias among the taxa examined, 11 microsatellite loci isolated from *P. maniculatus* can be confidently concluded to provide reliable estimates of genetic variation and evolutionary relationships within the *P. maniculatus* species group and at least to the evolutionary timescale represented by the divergence of *P. leucopus* from the *P. maniculatus* group lineage. These data support the more general conclusion that given the choice of an appropriate focal species, even relatively small sets of pure dinucleotide microsatellites can be expected to provide reliable population genetic information and systematic implications for taxa with divergence times dating at least to the Pleistocene.

CHAPTER IV

EVOLUTIONARY IMPLICATIONS OF MICROSATELLITE VARIATION IN THE *Peromyscus maniculatus* SPECIES GROUP

INTRODUCTION

Analysis of DNA microsatellites has rapidly become a popular approach for studies of population genetics and evolutionary relationships. The rapid rate of microsatellite mutation (Weber and Wong 1993) makes microsatellites more sensitive indicators of population genetic interactions and creates a greater probability of yielding differences between taxa separated for shorter evolutionary times than other markers such as allozymes and RFLP fragments. Comparative analyses of microsatellite variation should, therefore, be particularly well suited for the identification of cryptic species and for resolving the relationships among recently diverged taxa. However, due to the anticipated complications of microsatellites (e.g., ambiguities in the mutational process, ascertainment bias, homoplasy) only a very limited number of experimental studies have been performed to test the applicability of microsatellites for phylogenetic comparisons among species.

The highly corroborated phylogeny for most of the species in the *Peromyscus maniculatus* species group (fig. 1) provides a unique opportunity to test the applicability of microsatellites for genealogical inference. In an attempt to resolve the systematic

affinities of *P. maniculatus*, *P. keeni*, and *P. sejugis* and their relationships to the other taxa in the *P. maniculatus* species group (figs. 2 and 3), we conducted a comparative analysis of variation at 12 pure-repeat dinucleotide ($(CA)_{n=18\ to\ 27}$) DNA microsatellite markers isolated from *P. maniculatus* (Chirhart et al. 2000) for samples of each of the species in the *P. maniculatus* species group.

MATERIALS AND METHODS

Genomic DNA was isolated (Sambrook et al. 1989) from frozen liver tissue for 20 individuals each of *P. maniculatus* (Kansas; Ellis Co, 1 mi S, 3 mi W Hays); *P. keeni* (Washington; Okanogan Co; 12.5 mi W Mazama, Lone Fir Campground (n=9), Gray's Harbor Co, 3.0 mi N, 1.0 mi E Grisdale, Satsop Workcamp (n=11)); *P. sejugis* (Mexico: Baja California; Isla San Diego (n=13), Isla Santa Cruz (n=7)); *P. polionotus* (South Carolina; Lexington Co, 1 mi S Edmund, Bethel Church Field); *P. melanotis* (Mexico: Durango; 44 km SW Ojitos); *P. leucopus* (Texas: Wichita Co, 8.6 mi N., 1.0 mi. W. Iowa Park, (n=11), Archer Co, 4 mi. N. Windthorst (n=5), Robertson Co, 12 mi. N, 6 mi. W of Girvin TX (n=4). Detailed information for the specimens examined is presented in Appendix I. From the DNA of each of these individuals, the microsatellite genotype was determined for each of 12 loci isolated and characterized from *P. maniculatus* (Chirhart et al., 2000).

Alleles were amplified using the polymerase chain reaction (PCR) with fluorescently labeled primers. All PCR protocols and primer pairs were as described in

Chirhart et al. (2000) with the re-optimized annealing temperatures and thermo-cycles described in Chapter 2. All amplifications were performed on an OMN-E Hybaid thermocycler (Ashford, United Kingdom).

In order to obtain genotypic assignments for each locus, approximately 1.5 µl of the PCR product was mixed with 1.25 µl of deionized formamide, 0.25 µl of loading dye (Applied Biosystems, Inc., Foster City, CA), and 0.25 µl of GeneScan-400HD ROX Size Standard (Applied Biosystems, Inc., Foster City, CA). After denaturing at 95°C for 5 min, the samples were placed on ice for 5 min and 1.5 µl of each sample was loaded into the gel on an ABI 310 automated sequencer (Applied Biosystems, Inc., Foster City, CA). GENESCAN™ and GENOTYPER™ software (Applied Biosystems, Inc., Foster City, CA) was used to determine allele sizes by direct comparison against the internal ROX size standard.

Chord distance (D_C, Cavalli-Sfroza and Edwards 1967), Nei's distance (D_A, Nei et al. 1983), and proportion of shared alleles (D_{PS}, Bowcock et al. 1994) are each based on differences in allele frequencies between the populations (Goldstein and Pollock 1997) and have been shown to perform well for establishing the systematic relationships of closely related taxa (Goldstein et al. 1995, Goldstein and Clark 1995; Takezaki and Nei 1996; Harr et al. 1998b; Muir et al. 2000). As the species in the *P. maniculatus* group are closely related and recently diverged, these estimates of genetic distance were used to evaluate the microsatellite-based relationships among the taxa. The neighbor-joining algorithm in PAUP* (Saitou and Nei 1987, Swofford 1999) and Fitch-

Margoliash algorithm in PHYLIP (Fitch and Margoliash 1967, Felsenstein 1993) were used to construct dendograms from the molecular distances.

RESULTS

GenBank BLAST (blastn and blastx) searches of the flanking sequences for all of the loci failed to identify association with any previously described introns, coding regions or amino acid sequences. Eleven of the 12 microsatellite primers amplified corresponding products in all individuals of the five species examined; *Pml08* amplified a product only in the individuals of *P. maniculatus*. For this locus, two additional primer sets were constructed (using the computer program OLIGO 5.0, National Biosytems Inc., Plymouth, MN) such that the forward and reverse primers were internal or external to the original *Pml08* primer sequences (Chirhart et al. 2000), respectively. PCR amplifications using all combinations of three primer sets (encompassing a total of 226 bases of forward and 84 bases of reverse flanking sequence) amplified identical genotypes of *Pml08* for individuals of *P. maniculatus*, but failed to yield corresponding products from the DNA of any of the nonfocal species.

The number of alleles and ranges of allele sizes for each of the 12 loci in each of the species except *P. sejugis* (the samples of which represent two small-island populations) are presented in table 8. The 11 loci present in all species (and *Pml08* in *P. maniculatus*) were polymorphic (Appendix III, Appendix IV) with as few as 3 alleles observed at *Pml04* in *P. keeni* and as many as 18 alleles detected at *Pml09* in *P. melanotis*. The average number of alleles per species (calculated based on the number of

Table 8
The number of alleles and the range of allele lengths (in parentheses) at the 12 microsatellite loci in *P. maniculatus*, *P. keeni*, *P. polionotus*, *P. melanotis*, and *P. leucopus*. Average is the mean number of alleles over all loci present in each species. For each species except *P. maniculatus* these values are calculated excluding *Pml08*.

Locus	*P. maniculatus*	*P. keeni*	*P. polionotus*	*P. melanotis*	*P. leucopus*
Pml01	11 (145-173)	12 (145-173)	12 (151-197)	14 (141-187)	14 (151-197)
Pml02	16 (197-253)	7 (221-257)	8 (211-245)	13 (223-253)	13 (217-245)
Pml03	12 (229-263)	13 (241-277)	13 (241-277)	17 (223-263)	12 (225-261)
Pml04	10 (198-226)	3 (194-202)	10 (198-226)	12 (194-232)	12 (200-232)
Pml05	11 (186-222)	9 (198-222)	17 (194-250)	10 (186-216)	10 (186-214)
Pml06	14 (134-172)	15 (134-172)	10 (130-164)	14 (134-172)	12 (136-172)
Pml07	13 (241-265)	12 (239-265)	14 (233-263)	11 (233-261)	10 (241-261)
Pml08	13 (209-235)	-	-	-	-
Pml09	11 (192-246)	10(202-260)	9(218-246)	18(180-246)	9(200-250)
Pml10	10 (160-200)	6 (158-184)	14 (160-200)	12 (158-192)	14 (158-200)
Pml11	16 (220-258)	8 (224-252)	10 (234-254)	16 (218-254)	13 (220-266)
Pml12	11 (143-175)	6 (149-165)	5 (143-163)	8 (145-179)	13 (143-173)
Average	12.3	9.2	11.1	13.2	12.1

loci amplified) ranged from 9.2 to 13.2 (table 8). The overall polymorphism and per-locus variation were markedly lower in *P. sejugis* (table 9) than in the other species examined. Additionally, of the three species represented by samples from multiple localities, only *P. sejugis* showed evidence of genetic discontinuity among the localities. The *P. sejugis* from Isla San Diego exhibited one or two alleles per locus. Ten of the 11 loci in the *P. sejugis* from Isla Santa Cruz displayed one or two alleles. *Pml01* exhibited six alleles with the allele fixed in the sample from Isla San Diego (171) having a frequency of 0.58 and the other alleles having frequencies of 0.07 or 0.14.

DISCUSSION

With the exception of the *Pml08* locus and the variation observed for insular endemic samples of *P. sejugis*, the microsatellite variation was wholly consistent across the taxa in the *P. maniculatus* species group. Pair-wise comparisons of the numbers of alleles per locus were not significantly different (chi-square test, $p>0.05$) between *P. maniculatus* and *P. keeni*, *P. polionotus*, *P. melanotis*, and *P. leucopus* or in the pair-wise comparisons among any of these species. Additionally, the ranges of allele sizes were largely overlapping among all of the species examined (tables 8 and 9). Despite amplifications with the redesigned primers, the *Pml08* microsatellite was detected only from the DNA of the individuals of *P. maniculatus* (molecular evolutionary implications of the species-specific nature of the *Pml08* locus was discussed in Chapter 3).

Overall, the microsatellite data for *P. sejugis* are consistent with the implications of allozymic (Avise et al. 1974) karyotypic (Smith et al. 2000) and mtDNA data (Hogan

Table 9
The alleles and frequencies (in parentheses) at the 11 microsatellite loci in samples of *P. sejugis* from two islands. Average is the mean number of alleles for each population.

	Isla San Diego	*Isla Santa Cruz*
Pml01	-	161 (0.07)
	-	169 (0.07)
	171 (1.00)	171 (0.58)
	-	173 (0.07)
	-	175 (0.14)
	-	179 (0.07)
Pml02	221 (1.00)	221 (1.00)
Pml03	257 (0.85)	257 (0.79)
	261 (0.15)	261 (0.21)
Pml04	192 (0.08)	192 (0.07)
	194 (0.92)	194 (0.93)
Pml05	220 (1.00)	220 (1.00)
Pml06	152 (0.81)	152 (1.00)
	156 (0.19)	
Pml07	257 (1.00)	257 (1.00)
Pml09	260 (1.00)	260 (1.00)
Pml10	180 (1.00)	180 (1.00)
Pml11	224 (1.00)	224 (1.00)
Pml12	153 (1.00)	149 (1.00)
Average	1.27	1.64

et al. 1997) in suggesting the substantial impact of founder effect on this species and the lack of recent gene flow between its two island populations. For 10 of the 11 microsatellite loci, the number of alleles in *P. sejugis* was significantly lower (chi-square test, $p<0.05$) than that observed in each of the other species; the single exception involved the comparison to the uncharacteristically low number of alleles observed at the *Pml04* locus in the sample of *P. keeni* (table 8). For the two island populations of *P. sejugis*, six loci were monomorphic for the same allele, two loci were monomorphic in one of the two island populations (*Pml01* in the sample from Isla San Diego and *Pml06* in that from Isla Santa Cruz), two loci (*Pml03* and *Pml04*) were polymorphic for the same alleles at similar frequencies, and one locus (*Pml12*) was fixed for a different allele in the two island populations (table 9). In direct contradiction to this general pattern, the *Pml01* locus exhibited six alleles in the sample of seven individuals of *P. sejugis* from Isla Santa Cruz. Verification of this result was obtained by repeating the entire methodology and analysis of *Pml01* for DNA isolated from additional samples of the tissue for all of the specimens of *P. sejugis*. Considering the consistency of minimal variation in allozymes, chromosomes and the other microsatellite loci in both populations of *P. sejugis*, a history of significant founder effects and genetic drift appears to eliminate retention of ancestral polymorphism or post-isolation gene flow as explanations for the variation at the *Pml01* locus in the *P. sejugis* from Isla Santa Cruz. Alternative explanations implicate an extraordinarily rapid rate of molecular evolution for additional alleles at this locus in this population. Although the comparative analyses of allelic variation provide no other indication of heterogeneity in the allelic mutation

rate at the 11 microsatellites shared by all of the species examined (Chapter 3), the high level of polymorphism at *Pml01* in the *P. sejugis* from Isla Santa Cruz at the focal-species specific *Pml08* support the hypothesis that elevated levels mutation can arise suddenly for particular dinucleotide microsatellites within individual evolutionary lineages.

Given their potential for elucidating otherwise cryptic genealogical relationships among closely related species, numerous authors have addressed the practical and theoretical concerns relative to the comparative analysis of microsatellites among taxa at various levels of evolutionary divergence (for reviews see Amos 1999, Chambers and MacAvoy 2000, Schlötterer 2000, Schlötterer 2001). Two primary considerations in the application of microsatellite loci for systematic studies are the potential for ascertainment bias (Ellegren et al. 1995) on the accuracy of assessing genetic variation when comparing microsatellite loci isolated from one species (the focal species) to species at increasing evolutionary distances from the focal species and the number of microsatellite loci required to provide reliable genealogical relationships among the taxa being compared. In this study, the potential for ascertainment bias (and markedly different interlocus rates of mutation) was minimized by restricting the comparisons to a single type of microsatellite (pure dinucleotide repeats). Pure dinucleotide microsatellites were chosen because the taxa in the *P. maniculatus* species group have recently diverged and pure dinucleotide microsatellites have a particularly rapid rate of evolution (Chakraborty et al. 1997). Except for the restriction of *Pml08* to the focal species (*P. maniculatus*) and the data for *P. sejugis* (which are apparently biased by

founder effect and genetic drift), there was no evidence of significant allelic asymmetry in the application of the microsatellites from *P. maniculatus* to the other species in the *P. maniculatus* species group or to *P. leucopus* (Chapter 3). Tests for significant ascertainment bias included comparative analyses of PCR product sizes (mean, median, and modal lengths), number of alleles, observed and expected heterozygosities, and allele size distribution at each locus between *P. maniculatus* and each of the nonfocal species examined.

Although various studies have used microsatellite polymorphisms to address systematic questions (Meyer et al. 1995, Zardoya et al. 1996, Petren et al. 1997), the number of microsatellite loci required for accurate assessments of evolutionary relationships remains unresolved. Computer simulations have suggested that as many as 100 microsatellite loci may be required for reliable genealogical inferences among species (Takezaki and Nei 1996). Empirical studies, however, have obtained expected phylogenetic relationships from analyses of relatively few microsatellite loci (Harr et al. 1998a,b, Muir et al. 2000, Ritz et al. 2000). In this study, the interspecific genealogical reliability of the 11 microsatellite loci that occurred in all of the species was assessed by determining the extent to which results of systematic analyses conformed to the highly corroborated phylogeny for *P. maniculatus*, *P. polionotus*, *P. melanotis*, and *P. leucopus*. Neighbor-joining and Fitch-Margoliash analyses for each of the three distance estimates (D_C, D_A, and D_{PS}, table 10) among the individuals of *P. maniculatus*, *P. polionotus*, *P. melanotis*, and *P. leucopus* correctly grouped all individuals to species in a well-defined

Table 10
Genetic distances (D_{PS} above the diagonal, D_A above the diagonal in parenthesis, and D_C below the diagonal) for the pairwise comparisons of the alleles at the 11 microsatellites present in all samples of the species in the *Peromyscus maniculatus* group and *P. leucopus*.

	P. maniculatus	*P. keeni*	*P. sejugis* (SD)	*P. sejugis* (SC)	*P. polionotus*	*P. melanotis*	*P. leucopus*
P. maniculatus	-	0.580 (0.00)	0.862 (0.871)	0.844 (0.857)	0.568 (0.604)	0.598 (0.631)	0.598 (0.651)
P. keeni	0.398	-	0.703 (0.641)	0.703 (0.644)	0.657 (0.602)	0.668 (0.612)	0.732 (0.671)
P. sejugis (SD)	0.595	0.322	-	0.150 (0.134)	0.931 (0.850)	0.927 (0.850)	0.936 (0.852)
P. sejugis (SC)	0.574	0.327	0.086	-	0.936 (0.858)	0.919 (0.842)	0.920 (0.854)
P. polionotus	0.649	0.422	0.734	0.724	-	0.639 (0.524)	0.661 (0.606)
P. melanotis	0.661	0.412	0.692	0.699	0.575	-	0.570 (0.523)
P. leucopus	0.674	0.696	0.675	0.675	0.581		-

SD=*P. sejugis* from Isla San Diego

SC=*P. sejugis* from Isla San Cruz

tree identical to the highly corroborated phylogeny for these species (fig. 4). These results support the reliable utility of these microsatellites for both population genetic and genealogic studies among and within the species in the *P. maniculatus* group and suggest that relatively small sets of pure dinucleotide microsatellite loci can be confidently applied across taxa with divergence times dating to the late Pleistocene.

Given the lack of significant ascertainment bias and the correspondence of the microsatellite-based relationships to the corroborated phylogeny for the *P. maniculatus* group species, the interspecific D_C, D_A, and D_{PS} distances from the data for the 11 loci common to all of the species examined (table 10) were used to infer the genealogical relationships of the more problematical species, *P. keeni* and *P. sejugis*. Neighbor-joining and Fitch-Margoliash analyses for each of the distances yielded topologically identical trees with *P. keeni* (from the Pacific Northwest) and *P. sejugis* (from two small islands off the east coast of southern Baja California) together forming a sister group to *P. maniculatus* (fig. 6). Although surprising from a geographic point of view, the sister-group association of *P. keeni* and *P. sejugis* was also obtained from restriction site and preliminary sequence analyses of the ND3/ND4L/ND4 region of the mtDNA (Hogan et al. 1997). Two scenarios can be invoked to explain this relationship. *Peromyscus keeni* and *P. sejugis* may have shared a common ancestor after divergence from the lineage that gave rise to *P. maniculatus*. This common ancestor would have occupied a coastal range isolated west of the Cascade and Sierra Nevada Mountains. The current disjunct distributions of *P. keeni* and *P. sejugis* would have resulted from the extinction of the hypothetically intervening populations. The range from Washington south through Baja

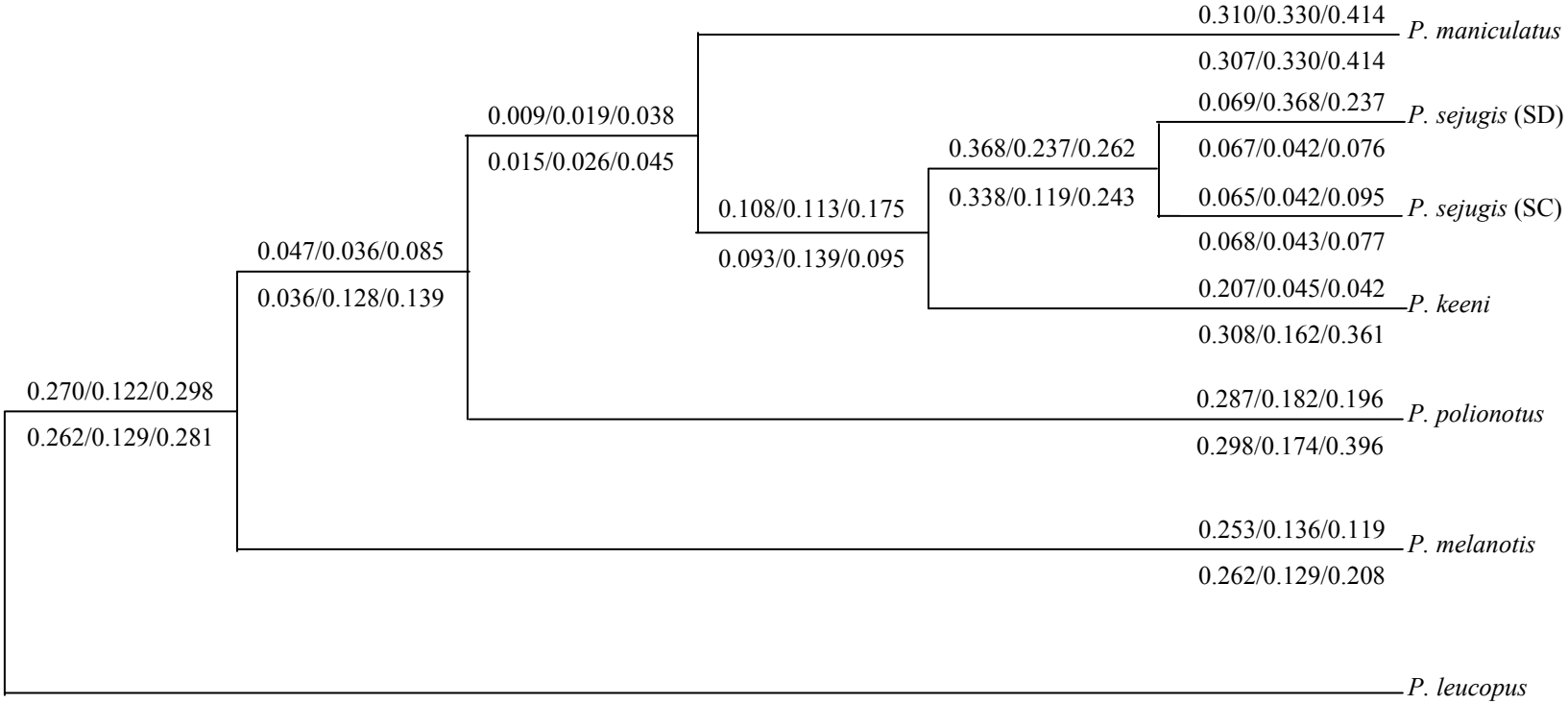

Fig. 6 – Microsatellite-based tree from the neighbor-joining and Fitch-Margoliash analyses of $D_{PS}/D_A/D_C$ for the species in the *P. maniculatus* species group. Branch lengths are $D_{PS}/D_A/D_C$ values based on the neighbor-joining (above the line) and Fitch-Margoliash (below the line) analyses.

California is, however, inhabited by deer mice currently recognized as representing 6 mainland and 17 insular subspecies of *P. maniculatus* (Hall 1981). Alternatively, *P. keeni* and *P. sejugis* may correspond to the pattern of centrifugal speciation (Brown 1957) and represent independent founder events from a *P. maniculatus* central stock. According to this hypothesis, the genetic similarities between *P. keeni* and *P. sejugis* would be the coincidental result of founder effects (and subsequent genetic drift) and the apparent sister-group association of *P. keeni* and *P. sejugis* would be a reflection of large-scale lineage sorting rather than true phylogenetic propinquity. This hypothesis predicts that the gene-level similarities between *P. sejugis* and *P. keeni* will prove to be retentions of plesiomorphic neutral alleles and that *P. maniculatus* will be characterized by elevated levels of polymorphisms for derived neutral alleles. Testing the ancestral-continuity versus centrifugal speciation hypotheses will, however, require a far greater knowledge of the distribution of genetic variation, particularly in *Peromyscus maniculatus* from Baja California and Pacific coastal North America.

CHAPTER V

SUMMARY AND CONCLUSIONS

Although nuclear DNA microsatellites have rapidly become the character of choice for studies spanning virtually all aspects of population and evolutionary genetics, many questions remain relative to the mutational basis of microsatellites and the constraints of the use of these as characters for either intra or interspecific comparisons. As the per-species isolation of microsatellite loci is laborious, the most popular approach is to employ microsatellites isolated from one (focal) species for analyses of variation and genealogy of other (nonfocal) species. Empirical data, however, indicate that this approach is subject to questionable reliability due to ascertainment bias exemplified by overall patterns of shorter alleles and lower polymorphism due to the *a priori* selective isolation of long and highly polymorphic microsatellite loci in the focal species.

The central premise of this research is that reliable population genetic and evolutionary applications of interspecific comparisons of microsatellites isolated from a focal species are constrained by variation in the types of microsatellites employed, the choice of an appropriate focal species, and the phylogenetic depth (total evolutionary time) encompassed by the taxonomic group being compared. To test this hypothesis, this study employed an experimental design that included analyses of a uniform category of microsatellite loci in a recently evolved group of mammals (the deer mice in the *P.*

maniculatus species group) for which there are ample data to specify the appropriate focal species (*P. maniculatus*) and for which there is a highly corroborated phylogeny.

Twelve pure dinucleotide microsatellite loci were isolated and characterized for *P. maniculatus* and used to make comparisons of variation within and among the species in the *P. maniculatus* group and the outgroup species *P. leucopus*. With the exception of one locus (*Pml08*) being unique to *P. maniculatus*, there was no significant ascertainment bias in allelic variation in any of the nonfocal mainland species in either the *P. maniculatus* species group (*P. keeni*, *P. polionotus*, *P. melanotis*) or *P. leucopus*. Phenetic analyses of the allelic data yielded inferred relationships that were entirely concordant with the *a priori* corroborated phylogeny for the species in the *P. maniculatus* species group. These results indicate that the 11 microsatellite loci isolated from *P. maniculatus* can be confidently concluded to provide reliable estimates of genetic variation and evolutionary relationships within the *P. maniculatus* species group and at least to the evolutionary timescale represented by the divergence of *P. leucopus* from the *P. maniculatus* group lineage and support the more general conclusion that given the choice of an appropriate focal species, even relatively small sets of pure dinucleotide microsatellites can be expected to provide reliable population genetic and genealogical implications for taxa with divergence times dating at least to the Pleistocene.

Given the lack of significant ascertainment bias, the 11 microsatellite loci common to all of the *P. maniculatus* group species were used to examine genetic variation in the two populations of the insular endemic species *P. sejugis*. These

analyses documented evidence of significant founder effect/genetic drift in the samples of *P. sejugis* and support previously described preliminary data for the sister-group relationship of *P. keeni* and *P. sejugis*. Two possible scenarios can explain this geographically improbable relationship. An historical ancestral continuity hypothesis suggests that *P. keeni* and *P. sejugis* shared a common ancestor after divergence from the lineage that gave rise to *P. maniculatus* and are true sister taxa. Alternatively, *P. keeni* and *P. sejugis* may correspond to the pattern of centrifugal speciation (Brown 1957), their genetic relatedness being the result of large-scale lineage sorting rather than true phylogenetic propinquity.

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APPENDIX I

SPECIMENS EXAMINED

ID number	*Genus species*	Specific Locality	County	State/ Country
GK4488	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK4489	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK4497	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK4498	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK4499	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK4500	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK5365	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK5366	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK5367	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK5368	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6063	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6064	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6164	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6226	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6227	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6235	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6236	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6524	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6525	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK6527	*P. maniculatus*	1 mi S, 3 mi W Hays	Ellis	KS
GK5689	*P. keeni*	Lone Fir Campgrounds; 12.5 mi W Mazama	Okanogan	WA
GK5690	*P. keeni*	Lone Fir Campgrounds; 12.5 mi W Mazama	Okanogan	WA
GK5691	*P. keeni*	Lone Fir Campgrounds; 12.5 mi W Mazama	Okanogan	WA
GK6060	*P. keeni*	Lone Fir Campgrounds; 12.5 mi W Mazama	Okanogan	WA
GK6061	*P. keeni*	Lone Fir Campgrounds; 12.5 mi W Mazama	Okanogan	WA
GK6085	*P. keeni*	Lone Fir Campgrounds; 12.5 mi W Mazama	Okanogan	WA
GK6086	*P. keeni*	Lone Fir Campgrounds; 12.5 mi W Mazama	Okanogan	WA
GK6211	*P. keeni*	Lone Fir Campgrounds; 12.5 mi W Mazama	Okanogan	WA
GK6212	*P. keeni*	Lone Fir Campgrounds; 12.5 mi W Mazama	Okanogan	WA
GK6218	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6219	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6240	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6241	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6244	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6245	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6240	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6241	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6246	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6271	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA
GK6272	*P. keeni*	Satsop Workcamp; 3.0 mi N, 1.0 mi E Grisdale	Gray's Harbor	WA

GK5462	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5463	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5464	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5465	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5466	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5467	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5468	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5469	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5470	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5471	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5472	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5473	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5474	*P. sejugis*	Isla San Diego-Gulf of California	Baja CA Sur	MEX
GK5477	*P. sejugis*	Isla Santa Cruz-Gulf of California	Baja CA Sur	MEX
GK5478	*P. sejugis*	Isla Santa Cruz-Gulf of California	Baja CA Sur	MEX
GK5479	*P. sejugis*	Isla Santa Cruz-Gulf of California	Baja CA Sur	MEX
GK5480	*P. sejugis*	Isla Santa Cruz-Gulf of California	Baja CA Sur	MEX
GK5481	*P. sejugis*	Isla Santa Cruz-Gulf of California	Baja CA Sur	MEX
GK5482	*P. sejugis*	Isla Santa Cruz-Gulf of California	Baja CA Sur	MEX
GK5483	*P. sejugis*	Isla Santa Cruz-Gulf of California	Baja CA Sur	MEX
GK5904	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5937	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5938	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5982	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5983	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5984	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5985	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5986	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5987	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5988	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5989	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK5990	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK6037	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK6038	*P. polionotus*	1 mi S Edmund, Bethel Church Field	Lexington	SC
GK6112	*P. polionotus*	1/4 mi from SCENA powerline and Edmund Rd.	Lexington	SC
GK6113	*P. polionotus*	1/4 mi from SCENA powerline and Edmund Rd.	Lexington	SC
GK6121	*P. polionotus*	1/4 mi from SCENA powerline and Edmund Rd.	Lexington	SC
GK6122	*P. polionotus*	1/4 mi from SCENA powerline and Edmund Rd.	Lexington	SC
GK6191	*P. polionotus*	1/4 mi from SCENA powerline and Edmund Rd.	Lexington	SC
GK6192	*P. polionotus*	1/4 mi from SCENA powerline and Edmund Rd.	Lexington	SC
TK 70754	*P. melanotis*	30 km SW Ojitos	Durango	MEX
TK 70755	*P. melanotis*	31 km SW Ojitos	Durango	MEX
TK 70756	*P. melanotis*	32 km SW Ojitos	Durango	MEX
TK 70757	*P. melanotis*	33 km SW Ojitos	Durango	MEX
TK 70758	*P. melanotis*	34 km SW Ojitos	Durango	MEX
TK 70759	*P. melanotis*	35 km SW Ojitos	Durango	MEX
TK 70760	*P. melanotis*	36 km SW Ojitos	Durango	MEX
TK 70761	*P. melanotis*	37 km SW Ojitos	Durango	MEX

TK 70762	*P. melanotis*	38 km SW Ojitos	Durango	MEX
TK 70763	*P. melanotis*	39 km SW Ojitos	Durango	MEX
TK 70764	*P. melanotis*	40 km SW Ojitos	Durango	MEX
TK 70765	*P. melanotis*	41 km SW Ojitos	Durango	MEX
TK 70766	*P. melanotis*	42 km SW Ojitos	Durango	MEX
TK 70767	*P. melanotis*	43 km SW Ojitos	Durango	MEX
TK 70768	*P. melanotis*	44 km SW Ojitos	Durango	MEX
TK 70810	*P. melanotis*	45 km SW Ojitos	Durango	MEX
TK 70949	*P. melanotis*	46 km SW Ojitos	Durango	MEX
TK 70950	*P. melanotis*	47 km SW Ojitos	Durango	MEX
TK 70951	*P. melanotis*	48 km SW Ojitos	Durango	MEX
TK 70952	*P. melanotis*	49 km SW Ojitos	Durango	MEX
GK5848	*P. leucopus*	Robertson Co.	Robertson	TX
GK5849	*P. leucopus*	Robertson Co.	Robertson	TX
GK5850	*P. leucopus*	Robertson Co.	Robertson	TX
GK5860	*P. leucopus*	12 mi. N., 6 mi. W of Girvin	Pecos	TX
GK5861	*P. leucopus*	13 mi. N., 6 mi. W of Girvin	Pecos	TX
GK5975	*P. leucopus*	4 mi. N. Windthorst	Archer	TX
GK5976	*P. leucopus*	5 mi. N. Windthorst	Archer	TX
GK5977	*P. leucopus*	6 mi. N. Windthorst	Archer	TX
GK5978	*P. leucopus*	7 mi. N. Windthorst	Archer	TX
GK6076	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6077	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6083	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6084	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6089	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6220	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6221	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6233	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6242	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6243	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX
GK6265	*P. leucopus*	8.6 mi N., 1.0 mi. W. Iowa Park	Wichita	TX

APPENDIX II

PML08 SEQUENCE

Sequence of *Pml08*. The original primer sequences (Chirhart et al. 2000) are in bold; the additional external and internal primers are designated as bold and underlined and bold and italics, respectively. The flanking sequence for the external primer set includes 226bp preceding and 84bp following the repeat sequence.

GATCCCTGGGGCTAGC**AGGCCAGCCAGTCTAGCTGA**ATGGGCCAGTGAGA

GAGCCTGTCTAAGAGAACAAGGTCAGAGGGGCTCGGGGG**AATGGCTCAGT**

CCTCTTCCTGCAGGACCTGAGT***TCAGATGCCGGTACCCACGG***AAGTTGACTC

ACAACAGTGTGTCATTTCAGATGTAGGTCTGACACTCTCTCCACATACCTGT

GCTGATATGCACATAACCAGCCCTACACACACACACCTACACACACACACAC

ACACACACACACACACACACACACACACACTTTAAAA***TAATAAAGATAAATC***TT

TAAAA**GAACAAGGTTGATAGCACCC**TAGGAACAATTGGTG**AAGCTGACAC**

CAGGCCTTCACCCACATGTGCAGAAAGACACACACACCCTAACCAGGAAG

AGCAAAAGGGCCACACAAAGAGCACTTAGTCTTTTTTGTTGGAAAACAAAA

AACTTNCCAGGTCTGGAGGTTTTTT

APPENDIX III

GENOTYPES

Per individual genotypes (in terms of allele lengths) at each of the 12 microsatellites examined.

INDIV. #	*Pml01*		*Pml02*		*Pml03*		*Pml04*		*Pml05*		*Pml06*		*Pml07*		*Pml08*		*Pml09*		*Pml10*		*Pml11*		*Pml12*	
P. maniculatus																								
GK4488	155	171	251	253	241	255	222	226	210	220	148	172	249	261	213	213	202	230	172	182	244	258	147	169
GK4489	165	171	245	247	247	255	198	220	216	220	156	158	243	255	221	231	200	230	182	184	228	252	149	149
GK4497	157	169	251	251	251	255	210	220	220	220	152	158	259	263	209	211	202	202	180	194	222	256	149	151
GK4498	169	173	229	239	241	263	218	222	194	218	142	148	247	251	221	221	230	244	180	180	240	252	147	169
GK4499	163	163	227	235	251	257	212	220	210	220	152	162	255	257	213	221	194	242	172	182	236	238	149	155
GK4500	169	173	219	245	229	261	198	220	198	220	162	164	243	245	219	221	224	246	182	184	244	248	159	169
GK5365	171	173	239	243	235	269	198	222	210	220	158	160	241	265	213	215	192	200	160	182	240	252	143	147
GK5366	165	171	219	223	241	255	220	224	202	220	146	146	257	261	221	221	242	246	172	182	244	254	151	169
GK5367	145	171	251	251	247	269	218	220	186	222	146	152	243	255	213	213	202	236	166	180	234	244	151	165
GK5368	153	171	239	247	255	255	220	220	200	220	146	162	249	261	221	231	236	242	180	200	230	252	149	155
GK6063	157	161	219	225	233	241	208	220	210	220	146	162	247	259	221	221	200	246	180	182	244	252	149	151
GK6064	161	169	251	251	247	255	218	218	216	222	150	156	249	257	213	229	236	242	180	184	220	244	161	175
GK6164	163	171	237	239	255	269	222	226	200	220	134	134	247	257	215	219	202	210	168	180	234	248	159	161
GK6226	151	161	247	251	251	255	220	220	210	220	146	172	253	255	223	235	200	200	172	182	248	250	143	145
GK6227	155	171	251	253	241	255	222	226	210	220	148	172	249	261	221	231	242	246	172	182	244	254	151	169
GK6235	171	171	239	251	239	255	216	220	216	222	154	160	241	265	213	227	192	200	180	182	244	256	147	169
GK6236	171	173	229	231	229	255	216	222	200	208	148	152	247	247	223	225	230	236	182	196	248	250	151	155
GK6524	155	171	215	239	255	269	208	216	220	220	152	156	241	257	211	213	202	246	180	194	244	252	169	175
GK6525	153	171	197	231	239	255	198	208	220	220	140	146	247	257	221	229	200	236	172	182	222	246	149	151
GK6527	165	171	219	223	241	255	220	224	202	220	146	146	257	261	233	235	202	230	172	182	244	258	147	169
P. keeni																								
GK5689	155	171	257	257	261	261	194	200	210	220	146	156	251	263	-	-	202	260	180	180	224	250	149	153
GK5690	165	171	241	243	269	275	200	200	216	220	156	158	253	257	-	-	202	202	170	170	238	250	149	149
GK5691	157	169	243	243	263	275	200	200	220	220	152	158	255	261	-	-	238	240	176	176	228	250	155	161
GK6060	169	173	241	241	269	269	200	200	210	220	142	148	249	257	-	-	234	234	180	180	234	250	149	161
GK6061	163	163	233	233	259	269	202	202	218	222	152	162	255	265	-	-	238	244	180	180	236	252	153	161
GK6085	169	173	243	243	269	269	200	200	198	216	162	164	259	261	-	-	238	244	180	180	236	242	163	165

	Pml01		*Pml02*		*Pml03*		*Pml04*		*Pml05*		*Pml06*		*Pml07*		*Pml08*		*Pml09*		*Pml10*		*Pml11*		*Pml12*	
GK6086	171	173	221	243	269	275	194	200	210	220	158	160	255	257	-	-	202	260	170	176	224	236	149	153
GK6211	165	171	221	221	257	261	194	194	210	220	146	146	239	241	-	-	254	260	158	162	224	250	153	153
GK6212	145	171	221	229	259	261	194	202	218	222	136	150	247	257	-	-	234	244	162	180	224	228	149	161
GK6218	153	171	221	233	269	269	200	202	220	220	148	148	253	257	-	-	238	240	176	180	224	236	149	149
GK6219	157	161	221	241	265	265	194	200	210	216	158	162	257	257	-	-	252	260	170	180	234	250	149	161
GK6240	161	169	229	241	249	253	200	200	216	222	150	156	251	261	-	-	202	220	170	176	234	250	149	153
GK6241	163	171	243	251	257	277	194	200	220	220	134	146	241	257	-	-	256	260	176	180	224	224	153	161
GK6244	151	161	241	243	269	269	194	200	218	220	146	172	249	261	-	-	260	260	176	180	236	250	149	153
GK6245	163	169	221	229	243	245	200	202	200	218	146	156	241	259	-	-	202	244	170	180	236	250	163	165
GK6240	171	171	221	229	269	275	200	202	200	216	154	160	259	261	-	-	254	260	176	180	236	250	153	153
GK6241	147	173	221	243	257	261	194	200	200	208	148	152	255	261	-	-	256	260	158	162	224	224	149	161
GK6246	155	171	241	243	259	259	200	202	220	220	152	156	241	265	-	-	238	238	170	180	236	250	153	163
GK6271	153	171	241	257	261	261	194	200	220	220	140	150	257	261	-	-	240	260	176	176	224	236	149	153
GK6272	155	161	221	241	267	275	194	194	200	214	142	156	251	255	-	-	252	260	180	184	224	234	149	153
P. sejugis																								
GK5462	171	171	221	221	257	257	192	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	153	153
GK5463	171	171	221	221	257	257	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	153	153
GK5464	171	171	221	221	261	261	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	153	153
GK5465	171	171	221	221	257	257	194	194	220	220	152	156	257	257	-	-	260	260	180	180	224	224	153	153
GK5466	171	171	221	221	261	261	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	153	153
GK5467	171	171	221	221	257	257	194	194	220	220	152	156	257	257	-	-	260	260	180	180	224	224	153	153
GK5468	171	171	221	221	257	257	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	153	153
GK5469	171	171	221	221	257	257	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	153	153
GK5470	171	171	221	221	257	257	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	153	153
GK5471	171	171	221	221	257	257	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	153	153
GK5472	171	171	221	221	257	257	192	194	220	220	152	156	257	257	-	-	260	260	180	180	224	224	153	153
GK5473	171	171	221	221	257	257	194	194	220	220	152	156	257	257	-	-	260	260	180	180	224	224	153	153
GK5474	171	171	221	221	257	257	194	194	220	220	152	156	257	257	-	-	260	260	180	180	224	224	149	149
GK5477	171	171	221	221	257	257	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	149	149
GK5478	175	175	221	221	257	257	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	149	149
GK5479	171	173	221	221	257	261	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	149	149
GK5480	161	171	221	221	257	257	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	149	149
GK5481	171	179	221	221	257	261	192	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	149	149
GK5482	171	171	221	221	257	261	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	149	149
GK5483	169	171	221	221	257	257	194	194	220	220	152	152	257	257	-	-	260	260	180	180	224	224	149	149

	Pml01		*Pml02*		*Pml03*		*Pml04*		*Pml05*		*Pml06*		*Pml07*		*Pml08*		*Pml09*		*Pml10*		*Pml11*		*Pml12*	
P. polionotus																								
GK5904	161	165	219	219	261	261	222	226	218	240	150	158	259	257	-	-	222	232	166	182	248	248	143	163
GK5937	163	171	219	219	269	275	198	220	222	222	130	160	251	247	-	-	224	242	182	182	248	248	143	145
GK5938	159	161	219	223	263	275	210	220	214	234	150	150	245	239	-	-	242	246	160	184	248	252	143	149
GK5982	155	173	219	219	269	269	218	222	206	228	150	150	255	255	-	-	218	246	174	184	242	242	143	143
GK5983	151	163	219	223	259	269	212	220	216	222	156	156	249	249	-	-	222	234	168	168	242	246	143	143
GK5984	161	165	219	223	269	269	198	220	202	212	130	152	241	233	-	-	224	232	162	194	234	238	143	145
GK5985	165	165	219	223	269	275	198	222	206	224	150	158	255	253	-	-	242	242	178	184	246	246	143	149
GK5986	151	161	219	223	257	261	220	224	226	250	130	156	255	241	-	-	242	242	178	184	250	250	145	145
GK5987	157	161	219	219	259	261	218	220	228	242	156	156	261	249	-	-	234	242	168	168	238	240	145	147
GK5988	161	165	219	219	269	269	220	220	194	200	158	160	255	243	-	-	238	246	178	178	234	242	145	163
GK5989	151	155	219	223	265	265	222	226	216	222	158	164	261	249	-	-	242	242	184	194	238	250	143	143
GK5990	161	165	219	219	249	253	218	218	216	222	150	158	261	253	-	-	218	218	168	178	238	248	143	149
GK6037	163	163	219	219	257	277	222	226	206	224	150	158	255	249	-	-	242	246	170	170	234	250	163	163
GK6038	153	165	239	239	269	269	220	220	216	218	134	134	261	233	-	-	242	246	168	168	234	246	143	145
GK6112	161	177	215	219	243	245	208	216	216	216	160	160	255	243	-	-	234	238	174	184	234	244	145	149
GK6113	161	171	211	213	269	275	216	220	202	212	156	156	259	257	-	-	242	246	190	200	254	254	143	147
GK6121	161	197	219	223	257	261	216	222	194	200	132	160	261	259	-	-	222	242	172	178	244	244	145	147
GK6122	157	171	213	213	259	259	208	216	210	214	130	156	263	259	-	-	228	228	180	200	244	254	147	147
GK6191	161	161	213	239	261	261	208	216	210	214	142	156	233	239	-	-	246	246	172	180	246	246	143	147
GK6192	157	173	221	245	267	275	208	216	216	222	158	158	259	249	-	-	246	246	180	184	240	240	143	147
P. melanotis																								
TK 70754	167	169	227	237	261	261	208	208	200	204	148	172	239	255	-	-	180	194	158	180	248	250	161	163
TK 70755	169	171	225	227	255	261	206	208	200	204	156	158	245	253	-	-	232	232	164	174	248	250	161	165
TK 70756	141	173	223	231	233	253	208	212	208	212	152	158	245	239	-	-	184	230	174	174	248	250	165	165
TK 70757	149	149	243	247	227	263	206	212	186	216	142	148	245	247	-	-	232	232	174	180	248	248	159	163
TK 70758	171	171	223	223	235	243	212	214	208	214	152	162	245	255	-	-	200	232	170	178	238	242	161	161
TK 70759	149	171	235	237	229	261	208	214	204	208	162	164	239	247	-	-	190	232	178	184	234	242	157	157
TK 70760	163	165	235	243	243	243	204	212	214	214	158	160	241	255	-	-	232	232	178	178	224	224	157	159
TK 70761	155	159	223	231	231	261	208	204	208	208	146	146	245	247	-	-	224	226	180	180	250	252	159	159
TK 70762	163	171	249	253	251	255	208	204	200	214	146	162	245	247	-	-	190	232	178	184	234	242	157	165
TK 70763	145	171	235	237	255	255	208	212	208	214	146	162	255	261	-	-	200	232	174	178	248	252	157	165
TK 70764	163	171	249	253	251	255	208	204	200	214	146	162	245	247	-	-	200	232	174	182	250	252	161	165
TK 70765	163	165	239	245	249	249	218	220	208	212	150	156	255	261	-	-	236	246	182	192	246	250	159	167
TK 70766	163	167	235	237	245	251	218	224	204	208	134	134	257	259	-	-	218	234	176	182	230	232	145	159
TK 70767	145	165	231	237	255	261	208	228	202	204	146	172	239	253	-	-	200	224	178	180	250	254	157	179

	Pml01		*Pml02*		*Pml03*		*Pml04*		*Pml05*		*Pml06*		*Pml07*		*Pml08*		*Pml09*		*Pml10*		*Pml11*		*Pml12*	
TK 70768	145	145	227	239	237	255	194	230	208	214	146	156	239	255	-	-	212	234	174	182	224	238	165	167
TK 70810	163	187	237	239	251	259	218	228	210	214	154	160	241	255	-	-	230	232	180	190	248	250	159	161
TK 70949	163	169	237	239	257	259	204	212	206	212	148	152	239	247	-	-	190	232	174	182	236	220	159	163
TK 70950	165	167	223	233	245	257	208	212	204	212	152	156	253	255	-	-	202	204	174	178	234	250	161	163
TK 70951	173	179	223	225	253	259	228	232	206	208	140	150	233	249	-	-	208	232	174	178	218	228	159	165
TK 70952	169	181	235	237	223	231	204	218	204	214	142	156	245	245	-	-	232	242	172	174	232	240	161	167
P. leucopus																								
GK5848	157	173	217	239	255	255	206	216	200	214	152	154	255	257	-	-	230	250	160	180	260	260	151	159
GK5849	163	163	243	245	253	255	210	214	188	202	150	150	253	253	-	-	204	204	176	196	258	266	145	161
GK5850	161	165	239	245	239	255	208	212	192	200	148	172	253	257	-	-	204	210	158	180	252	260	155	165
GK5860	151	163	239	245	225	231	208	220	202	202	150	172	241	261	-	-	210	210	180	180	242	254	145	145
GK5861	161	165	217	233	255	261	200	216	200	206	152	160	255	255	-	-	210	250	180	194	242	254	145	171
GK5975	161	161	239	239	247	249	208	212	188	188	166	172	247	259	-	-	204	222	180	180	240	260	157	165
GK5976	163	171	227	241	255	261	208	222	188	202	154	172	255	257	-	-	210	242	176	196	232	252	143	171
GK5977	159	161	243	245	247	249	200	216	186	188	144	160	253	255	-	-	242	250	190	198	238	252	145	173
GK5978	161	165	241	241	249	251	206	216	202	212	166	172	251	255	-	-	204	210	180	184	252	252	145	169
GK6076	165	165	233	233	255	255	208	220	212	214	152	154	253	257	-	-	200	210	180	180	224	260	159	159
GK6077	161	165	239	245	247	251	208	212	188	214	144	166	247	253	-	-	210	216	186	194	248	252	159	165
GK6083	151	161	225	231	227	243	208	214	202	202	150	162	253	255	-	-	230	242	160	176	258	266	145	169
GK6084	165	179	243	245	231	255	210	214	188	200	136	150	247	255	-	-	202	204	176	196	228	260	157	163
GK6089	161	171	223	239	253	255	228	232	188	202	150	150	243	245	-	-	210	242	180	196	232	252	159	159
GK6220	153	165	235	239	255	255	212	216	212	214	142	150	253	255	-	-	210	210	194	200	242	254	143	155
GK6221	161	165	227	241	255	261	210	214	202	214	166	172	243	245	-	-	204	222	180	180	224	252	145	161
GK6233	155	173	229	239	247	249	208	220	210	214	150	150	247	247	-	-	222	242	168	170	220	254	157	165
GK6242	167	169	225	241	231	257	220	228	188	188	148	172	251	255	-	-	216	250	176	196	258	266	143	155
GK6243	161	197	217	233	249	251	218	232	194	202	152	154	253	255	-	-	210	210	176	192	228	238	153	161
GK6265	157	171	221	233	233	247	210	214	200	212	150	170	251	255	-	-	204	210	180	200	260	260	155	171

APPENDIX IV

ALLELE FREQUENCIES

Allele Frequencies and number of occurrences at each of the 12 microsatellites in each of the species samples examined.

P. maniculatus

Pml01			*Pml02*			*Pml03*			*Pml04*			*Pml05*			*Pml06*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
145	1	0.025	197	1	0.025	229	2	0.05	198	4	0.1	186	1	0.025	134	2	0.05
151	1	0.025	215	1	0.025	233	1	0.025	208	3	0.075	194	1	0.025	140	1	0.025
153	2	0.05	219	4	0.1	235	1	0.025	210	1	0.025	198	1	0.025	142	1	0.025
155	3	0.075	223	2	0.05	239	2	0.05	212	1	0.025	200	3	0.075	146	9	0.225
157	2	0.05	225	1	0.025	241	6	0.15	216	3	0.075	202	2	0.05	148	4	0.1
161	3	0.075	227	1	0.025	247	2	0.05	218	4	0.1	208	1	0.025	150	2	0.05
163	3	0.075	229	2	0.05	251	2	0.05	220	13	0.325	210	6	0.15	152	5	0.125
165	3	0.075	231	2	0.05	255	15	0.375	222	6	0.15	216	3	0.075	154	1	0.025
169	4	0.1	235	1	0.025	257	2	0.05	224	2	0.05	218	1	0.025	156	2	0.05
171	14	0.35	237	2	0.05	261	2	0.05	226	3	0.075	220	18	0.45	158	3	0.075
173	4	0.1	239	6	0.15	263	1	0.025				222	3	0.075	160	2	0.05
			243	1	0.025	269	4	0.1							162	4	0.1
			245	2	0.05										164	1	0.025
			247	2	0.05										172	3	0.075
			251	10	0.25												
			253	2	0.05												

P. maniculatus (cont.)

Pml07			*Pml08*			*Pml09*			*Pml10*			*Pml11*			*Pml12*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
241	3	0.075	209	1	0.025	192	2	0.05	160	2	0.05	220	1	0.025	143	2	0.05
243	3	0.075	211	2	0.05	194	1	0.025	166	1	0.025	222	2	0.05	145	1	0.025
245	1	0.025	213	9	0.225	200	7	0.175	168	1	0.025	228	1	0.025	147	5	0.125
247	6	0.15	215	2	0.05	202	7	0.175	172	7	0.175	230	1	0.025	149	7	0.175
249	4	0.1	219	2	0.05	210	1	0.025	180	10	0.25	234	2	0.05	151	7	0.175
251	1	0.025	221	12	0.3	224	1	0.025	182	13	0.325	236	1	0.025	155	3	0.075
253	1	0.025	223	2	0.05	230	5	0.125	184	2	0.05	238	1	0.025	159	2	0.05
255	4	0.1	225	1	0.025	236	5	0.125	194	2	0.05	240	2	0.05	161	2	0.05
257	7	0.175	227	1	0.025	242	5	0.125	196	1	0.025	244	10	0.25	165	1	0.025
259	2	0.05	229	2	0.05	244	1	0.025	200	1	0.025	246	1	0.025	169	8	0.2
261	5	0.125	231	3	0.075	246	5	0.125				248	4	0.1	175	2	0.05
263	1	0.025	233	1	0.025							250	2	0.05			
265	2	0.05	235	2	0.05							252	6	0.15			
												254	2	0.05			
												256	2	0.05			
												258	2	0.05			

P. keeni

Pml01			*Pml02*			*Pml03*			*Pml04*			*Pml05*			*Pml06*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
145	1	0.025	221	10	0.25	243	1	0.025	194	12	0.3	198	1	0.025	134	1	0.025
147	1	0.025	229	4	0.1	245	1	0.025	200	21	0.525	200	4	0.1	136	1	0.025
151	1	0.025	233	3	0.075	249	1	0.025	202	7	0.175	208	1	0.025	140	1	0.025
153	2	0.05	241	9	0.225	253	1	0.025				210	5	0.125	142	2	0.05
155	3	0.075	243	10	0.25	257	3	0.075				214	1	0.025	146	6	0.15
157	2	0.05	251	1	0.025	259	4	0.1				216	5	0.125	148	4	0.1
161	4	0.1	257	3	0.075	261	7	0.175				218	4	0.1	150	3	0.075
163	4	0.1				263	1	0.025				220	16	0.4	152	4	0.1
165	2	0.05				265	2	0.05				222	3	0.075	154	1	0.025
169	5	0.125				267	1	0.025							156	6	0.15
171	11	0.275				269	12	0.3							158	4	0.1
173	4	0.1				275	5	0.125							160	2	0.05
						277	1	0.025							162	3	0.075
															164	1	0.025

P. keeni (cont.)

Pml07			*Pml08*			*Pml09*			*Pml10*			*Pml11*			*Pml12*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
239	1	0.025	-	-	-	202	6	0.15	158	2	0.05	224	11	0.275	149	14	0.35
241	4	0.1				220	1	0.025	162	2	0.05	228	2	0.05	153	13	0.325
247	2	0.05				234	3	0.075	170	7	0.175	234	3	0.075	155	1	0.025
249	2	0.05				238	6	0.15	176	10	0.25	236	9	0.225	161	7	0.175
251	2	0.05				240	3	0.075	180	17	0.425	238	1	0.025	163	3	0.075
253	2	0.05				244	4	0.1	184	2	0.05	242	1	0.025	165	2	0.05
255	5	0.125				252	2	0.05				250	11	0.275			
257	9	0.225				254	2	0.05				252	2	0.05			
259	3	0.075				256	2	0.05									
261	7	0.175				260	11	0.275									
263	1	0.025															
265	2	0.05															

P. sejugis (Isla San Diego)

Pml01			*Pml02*			*Pml03*			*Pml04*			*Pml05*			*Pml06*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
171	26	1.00	221	26	1.00	257	22	0.85	192	2	0.08	220	26	1.00	152	21	0.81
						261	4	0.15	194	24	0.92				156	5	0.19

Pml07			*Pml08*			*Pml09*			*Pml10*			*Pml11*			*Pml12*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
257	26	1.00	-	-	-	260	26	1.00	180	26	1.00	224	26	1.00	153	26	1.00

P. sejugis (Isla Santa Cruz)

Pml01			*Pml02*			*Pml03*			*Pml04*			*Pml05*			*Pml06*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
161	1	0.07	221	14	1.00	257	11	0.79	192	1	0.07	220	14	1.00	152	14	1.00
169	1	0.07				261	3	0.21	194	13	0.93						
171	8	0.58															
173	1	0.07															
175	2	0.14															
179	1	0.07															

Pml07			*Pml08*			*Pml09*			*Pml10*			*Pml11*			*Pml12*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
257	14	1.00	-	-	-	260	14	1.00	180	14	1.00	224	40	1.00	149	14	1.00

P. polionotus

Pml01			*Pml02*			*Pml03*			*Pml04*			*Pml05*			*Pml06*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
151	3	0.075	211	1	0.025	243	1	0.025	198	3	0.075	194	2	0.05	130	3	0.075
153	1	0.025	213	3	0.075	245	1	0.025	208	4	0.1	200	2	0.05	132	1	0.025
155	2	0.05	215	1	0.025	249	1	0.025	210	1	0.025	202	2	0.05	134	2	0.05
157	3	0.075	219	22	0.55	253	1	0.025	212	1	0.025	206	3	0.075	142	1	0.025
159	1	0.025	221	2	0.05	257	3	0.075	216	6	0.15	210	2	0.05	150	8	0.2
161	12	0.3	223	7	0.175	259	4	0.1	218	4	0.1	212	2	0.05	152	2	0.05
163	4	0.1	239	2	0.05	261	7	0.175	220	11	0.275	214	3	0.075	156	9	0.225
165	7	0.175	245	2	0.05	263	1	0.025	222	6	0.15	216	7	0.175	158	8	0.2
171	3	0.075				265	2	0.05	224	1	0.025	218	2	0.05	160	5	0.125
173	2	0.05				267	1	0.025	226	3	0.075	222	6	0.15	164	1	0.025
177	1	0.025				269	12	0.3				224	2	0.05			
197	1	0.025				275	5	0.125				226	1	0.025			
						277	1	0.025				228	2	0.05			
												234	1	0.025			
												240	1	0.025			
												242	1	0.025			
												250	1	0.025			

P. polionotus (cont.)

Pml07			*Pml08*			*Pml09*			*Pml10*			*Pml11*			*Pml12*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
233	2	0.05	-	-	-	218	3	0.075	160	2	0.05	234	5	0.125	143	16	0.4
239	2	0.05				222	3	0.075	162	1	0.025	238	4	0.1	145	9	0.225
241	2	0.05				224	2	0.05	166	1	0.025	240	2	0.05	147	7	0.175
243	2	0.05				228	2	0.05	168	7	0.175	242	4	0.1	149	4	0.1
245	1	0.025				232	2	0.05	170	2	0.05	244	4	0.1	163	4	0.1
247	2	0.05				234	3	0.075	172	2	0.05	246	6	0.15			
249	6	0.15				238	2	0.05	174	2	0.05	248	6	0.15			
251	1	0.025				242	13	0.325	178	6	0.15	250	4	0.1			
253	2	0.05				246	10	0.25	180	2	0.05	252	2	0.05			
255	7	0.175							182	3	0.075	254	3	0.075			
257	2	0.05							184	7	0.175						
259	5	0.125							190	1	0.025						
261	5	0.125							194	2	0.05						
263	1	0.025							200	2	0.05						

P. melanotis

Pml01			*Pml02*			*Pml03*			*Pml04*			*Pml05*			*Pml06*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
141	1	0.025	223	6	0.15	223	1	0.025	194	2	0.05	186	1	0.025	134	2	0.05
145	4	0.1	225	2	0.05	227	1	0.025	204	6	0.15	200	4	0.1	140	1	0.025
149	3	0.075	227	2	0.05	229	1	0.025	206	2	0.05	202	1	0.025	142	2	0.05
155	1	0.025	231	2	0.05	231	2	0.05	208	11	0.275	204	7	0.175	146	7	0.175
159	1	0.025	233	2	0.05	233	1	0.025	212	7	0.175	206	2	0.05	148	3	0.075
163	7	0.175	235	5	0.125	235	1	0.025	214	2	0.05	208	10	0.25	150	2	0.05
165	4	0.1	237	8	0.2	237	1	0.025	218	3	0.075	210	2	0.05	152	4	0.1
167	3	0.075	239	4	0.1	243	3	0.075	220	2	0.05	212	3	0.075	154	1	0.025
169	4	0.1	243	2	0.05	245	2	0.05	224	1	0.025	214	9	0.225	156	5	0.125
171	7	0.175	245	2	0.05	249	2	0.05	228	2	0.05	216	1	0.025	158	3	0.075
173	2	0.05	247	1	0.025	251	4	0.1	230	1	0.025				160	2	0.05
179	1	0.025	249	2	0.05	253	2	0.05	232	1	0.025				162	5	0.125
181	1	0.025	253	2	0.05	255	7	0.175							164	1	0.025
187	1	0.025				257	2	0.05							172	2	0.05
						259	3	0.075									
						261	6	0.15									
						263	1	0.025									

P. melanotis (cont.)

Pml07			*Pml08*			*Pml09*			*Pml10*			*Pml11*			*Pml12*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
233	1	0.025	-	-	-	180	1	0.03	158	1	0.025	218	1	0.025	145	1	0.025
239	6	0.15				184	1	0.03	164	1	0.025	220	1	0.025	157	6	0.15
241	2	0.05				190	3	0.08	170	2	0.05	224	3	0.075	159	9	0.225
245	9	0.225				194	1	0.03	172	1	0.025	228	1	0.025	161	8	0.2
247	6	0.15				200	2	0.05	174	11	0.275	230	1	0.025	163	4	0.1
249	1	0.025				202	1	0.03	176	1	0.025	232	2	0.05	165	8	0.2
253	2	0.05				204	1	0.03	178	9	0.225	234	3	0.075	167	3	0.075
255	8	0.2				208	1	0.03	180	6	0.15	236	1	0.025	179	1	0.025
257	2	0.05				212	1	0.03	182	4	0.1	238	2	0.05			
259	1	0.025				218	1	0.03	184	2	0.05	240	1	0.025			
261	2	0.05				224	2	0.05	190	1	0.025	242	2	0.05			
						226	1	0.03	192	1	0.025	246	1	0.025			
						230	2	0.05				248	7	0.175			
						232	15	0.38				250	9	0.225			
						234	2	0.05				252	3	0.075			
						236	1	0.03				254	2	0.05			
						242	2	0.05									
						246	2	0.05									

P. leucopus

Pml01			*Pml02*			*Pml03*			*Pml04*			*Pml05*			*Pml06*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
151	2	0.05	217	2	0.05	225	1	0.025	200	2	0.05	186	1	0.025	136	1	0.025
153	1	0.025	221	2	0.05	227	1	0.025	206	2	0.05	188	10	0.25	142	1	0.025
155	1	0.025	223	1	0.025	231	3	0.075	208	8	0.2	192	1	0.025	144	2	0.05
157	2	0.05	225	2	0.05	233	1	0.025	210	4	0.1	194	1	0.025	148	2	0.05
159	1	0.025	227	2	0.05	239	1	0.025	212	4	0.1	200	5	0.125	150	11	0.275
161	11	0.275	229	1	0.025	243	1	0.025	214	5	0.125	202	10	0.25	152	4	0.1
163	4	0.1	231	1	0.025	247	5	0.125	216	5	0.125	206	1	0.025	154	4	0.1
165	9	0.225	233	5	0.125	249	5	0.125	218	2	0.05	210	2	0.05	160	2	0.05
167	1	0.025	235	1	0.025	251	2	0.05	220	3	0.075	212	3	0.075	162	1	0.025
169	1	0.025	239	9	0.225	253	2	0.05	222	1	0.025	214	6	0.15	166	4	0.1
171	3	0.075	241	5	0.125	255	13	0.325	228	2	0.05				170	1	0.025
173	2	0.05	243	3	0.075	257	2	0.05	232	2	0.05				172	7	0.175
179	1	0.025	245	6	0.15	261	3	0.075									
197	1	0.025															

P. leucopus (cont.)

Pml07			*Pml08*			*Pml09*			*Pml10*			*Pml11*			*Pml12*		
Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.	Allele	Count	Freq.
241	1	0.025	-	-	-	200	1	0.03	158	1	0.025	220	1	0.025	143	2	0.05
243	2	0.05				202	2	0.05	160	2	0.05	224	2	0.05	145	8	0.2
245	2	0.05				204	8	0.2	168	1	0.025	228	2	0.05	151	1	0.025
247	5	0.125				210	14	0.35	170	2	0.05	232	2	0.05	153	2	0.05
251	3	0.075				216	2	0.05	176	6	0.15	238	2	0.05	155	4	0.1
253	9	0.225				222	2	0.05	180	14	0.35	240	1	0.025	157	3	0.075
255	12	0.3				230	2	0.05	184	1	0.025	242	3	0.075	159	6	0.15
257	4	0.1				242	5	0.13	186	1	0.025	248	1	0.025	161	3	0.075
259	1	0.025				250	4	0.1	190	1	0.025	252	8	0.2	163	2	0.05
261	1	0.025							192	1	0.025	254	4	0.1	165	4	0.1
									194	2	0.05	258	3	0.075	169	2	0.05
									196	5	0.125	260	8	0.2	171	2	0.05
									198	1	0.025	266	3	0.075	173	1	0.025
									200	2	0.05						

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Texas Society of Mammalogists, Texas Society of Mammalogy Award, 2002.
Texas A&M University Department of Biology Poster Contest, Third Place. 2002
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PUBLICATIONS:

Chirhart, S.E., R.L. Honeycutt and I.F. Greenbaum. 2000. Microsatellite markers for the deer mouse *Peromyscus maniculatus*. Mol Ecol 9:1668-1671

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Manuscripts Submitted:

Chirhart, S.E., R.L. Honeycutt and I.F. Greenbaum. The use of microsatellites in nonfocal species: assessment of ascertainment bias and implications of molecular evolution in microsatellites isolated from *Peromyscus maniculatus* (submitted to Molecular Biology and Evolution)